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Cardiovascular Consequences When Nitric Oxide and Lipid Signaling Converge:

Convergence of NO and lipid signaling

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Abstract

The identification of nitric oxide (•NO) as an endogenously produced free radical mediator of endothelial-dependent relaxation and host defense has fundamentally changed concepts of cell signal transduction. Ligand-receptor oriented paradigms of cell signaling were originally centered on the concept of a high affinity and specific interaction between a ligand and its receptor, resulting in the activation of secondary signaling events such as gene expression or modulation of catalytic protein function. While •NO ligation of the heme iron of soluble guanylate cyclase is consistent with this perspective, the readily diffusible and broadly reactive •NO is increasingly appreciated to react with a vast array of target molecules that mediate paracrine vasodilator actions, inhibition of thrombosis and neointimal proliferation and both pro- and anti-inflammatory signaling reactions that are not affected by inhibitors of soluble guanylate cyclase. There is an expanding array of functionally significant “off target” collateral reactions mediated by •NO that are guanylate cyclase-independent and rather are dictated by anatomic distribution and the formation of secondary •NO-derived species. These reactions are a critical element of redox-regulated signaling and are addressed herein in the context of the oxidation of unsaturated fatty acids to vascular and inflammatory signaling mediators. Because of their abundance and the intrinsic reactivity of unsaturated lipid intermediates and eicosanoid metabolism enzymes with •NO and other oxides of nitrogen, lipid signaling mechanisms are a significant target for regulation by •NO in the vascular compartment. This convergence of •NO and lipid signaling pathways thus adds another level of regulation to physiological responses such as vasodilation, thrombosis and inflammation. Herein, interactions between •NO and lipid signaling events are placed in the context of cardiovascular regulation.

Keywords

nitric oxide; lipids; redox reaction; fatty acids; cardiovascular

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Nitric Oxide – Synthesis and Biological Effects

Nitric oxide synthase isoforms

Nitric oxide (•NO) is generated by three distinct subtypes of •NO synthase (NOS) isoenzymes that oxidize L-arginine to L-citrulline. [1] These can be subdivided into the constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS) and the inducible isoenzyme iNOS. [2] iNOS is strongly induced by a series of inflammatory stimuli such as endotoxin, interleukin-1β, interferon γ and tumor necrosis factor α. Inducible NOS was first reported to be present in macrophages [3] but has subsequently also been detected in vascular cell types including endothelial cells, [4] smooth muscle cells, [5] platelets [6] and myocytes. [7] In addition to its inducibility, iNOS differs from other NOS isoenzymes in that it does not require an increase in intracellular Ca^{2+}-concentration for catalytic activation. Although net extents of •NO production can exceed that of the constitutive isoenzymes up to 1000-fold it does not exhibit a greater rate of catalysis. Rather this phenomenon can be explained by higher protein concentrations of iNOS.[8–10]

Signal transduction by nitric oxide

Signaling reactions induced by •NO differ considerably from other signaling mediators. The predominant and best characterized physiological action of •NO is the formation of an Fe-nitrosyl intermediate with the heme iron of soluble guanylate cyclase (sGC), inducing sGC conformational changes and the activation of catalytic activity that in turn increases rates of production of cyclic guanosine monophosphate (cGMP). [11] The signaling actions of NOS enzymes are then propagated by activating cGMP-dependent ion channels, protein kinases and phosphodiesterases. [12] Via these effector proteins, •NO mediates alterations in intracellular Ca^{2+} levels, smooth muscle relaxation and the regulation of blood pressure. [13–15] In addition, this signaling axis mediates neurotransmission and inhibits both platelet aggregation and leukocyte function. [16]

Non-sGC/cGMP-dependent signaling by •NO

Because of the small molecular radius of •NO, its unpaired electron and a propensity to undergo reactions with diverse metal centers, molecular oxygen and oxygen-derived species, signaling reactions of •NO can also be induced by metal ligation and the post-translational modification of a broad population of susceptible target proteins. Consequently, this redox-related •NO signaling will be conveyed by changes in local •NO concentration and the presence of other redox intermediates that •NO can react with. [17] This introduces a panoply of additional sGC/cGMP-independent mechanisms whereby •NO can regulate inflammatory and vascular function, via reactions that are influenced by the protein reactivity of •NO and its metabolites.

While the major oxidation products of •NO present in tissues and excreted in urine are nitrite (NO_2^-) and nitrate (NO_3^-), •NO also reacts with molecular oxygen at relatively low rates and much more rapidly with a variety of radical species possessing unpaired electrons, yielding oxides of nitrogen (NO_x) sometimes referred to as reactive nitrogen species (RNS, Figure 1). Thus, •NO reacts with one of the two unpaired electrons of molecular oxygen to ultimately yield two molecules of nitrogen dioxide ($•NO_2$), a reaction shown to be of relevance in vivo. [18] Then, •NO can react further with $•NO_2$ to form the unstable nitrosating (addition of an NO-group) and nitrating (addition of an NO_2-group) species dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4). Dinitrogen trioxide yields nitrosonium ion (NO^+) and other metabolic reactions of •NO can yield nitroxyl (HNO), both shown to exert significant bioactivity. [19] Finally, in the context of redox signaling, a key metabolite of •NO is peroxynitrite ($ONOO^-$), formed by the kinetically fast reaction of •NO with superoxide ($O_2•^-$). [20] Of note, $ONOO^-$ is in itself a highly oxidizing species and has a relatively long aqueous half-life of

about 1.0 second. Moreover, reactions of $ONOO^-$ with H^+ or CO_2 give rise to secondary species such as hydroxyl radical (•OH), carbonate radical (HCO_3•) and •NO_2.[21]

Both •NO and •NO-derived RNS can transduce cell signaling by modulating the actions of biomolecules via a variety of chemical modifications, with the post-translational modification of proteins playing the most important role. Thus, S-nitrosation of cysteine thiols by •NO, a reaction mediated by a variety of •NO and thiol intermediates [32, 33] leads to a) the creation of a more stable reservoir for •NO and subsequent mitigation of sGC/cGMP-independent signaling and b) direct alterations in protein structure and function. [34] For example, S-nitrosation inhibits the activity of signaling cascades such as mitogen-activated protein kinase (MAPK), [35] Src kinases, [36] caspases, [37] protein tyrosine phosphatases [38] or the NOS-activity regulating enzyme dimethylarginine dimethylaminohydrolase (DDAH) [39] and modulates membrane receptors [40] and transcription factors, such as hypoxia-inducible factor-1α[41] and nuclear factor κB (NFκB).[42]

Via different mechanisms, the •NO-derived oxidizing and nitrating species $ONOO^-$ induces a variety of structural modifications to protein targets. [17] This includes the oxidation of thiols and the nitration of tyrosine residues, resulting in a broad array of cell signaling consequences. For example, $ONOO^-$ inhibits NFκB signaling [43] and multiple phosphotyrosine-dependent signaling mechanisms including MAPK [44] and the protein kinase B (Akt) pathways. [45] Cysteine thiols are very susceptible to oxidation by $ONOO^-$, as are Fe-S complexes and the ferrous heme of transition metal center containing proteins, all reactions that can contribute to the inhibition of mitochondrial respiratory chain activity [46] and iNOS. [47]

In addition to multifaceted reactions with proteins, •NO and its products play an important role in the oxidation and downstream signaling actions of lipids. [48] For example, •NO potently terminates the propagation of lipid peroxidation reactions, [30, 49, 50] while concomitantly forming hydroperoxide, epoxide and nitrated byproducts. [30] Under other conditions where reactive oxygen species (ROS) are abundant, •NO can alternatively initiate lipid oxidation via the generation of $ONOO^-$, [51, 52] resulting in the formation of lipid hydroperoxide, conjugated diene, aldehyde and isoprostane derivatives that can function as secondary signaling mediators.

Moreover, •NO and its products can regulate gene expression of enzymes of eicosanoid synthesis and, by direct protein interactions, modulate the enzymatic generation of lipid mediators while at the same time becoming catalytically consumed. The following sections expand on these events and underscore how the enzymatic and non-enzymatic generation of lipid mediators are modulated by •NO and •NO-derived species, resulting in altered vascular and inflammatory signaling.

Nitric Oxide Modulation of Enzymatically-Generated Lipid Mediators

Nitric oxide and prostaglandins

Prostanoid biosynthesis—Prostaglandins (PGs) are lipid mediators that play a key role in modulating vascular function. They are derived from the 20-carbon tetra-unsaturated fatty acid, arachidonic acid, by prostaglandin H synthase (PGHS) in a two-step reaction. Arachidonic acid is first converted into the unstable cyclic endoperoxide PGG_2 by the cyclooxygenase activity of PGHS. Subsequently, the peroxidase activity of PGHS cleaves the peroxide, which leads to the unstable endoperoxide PGH_2. Isomerization of this endoperoxide by specific enzymes finally yields multiple biologically active prostanoids such as thromboxanes and I, E, D F series prostaglandins. Physiologically, the signaling of vasorelaxant, antithrombotic prostanoids overrides those of their vasoconstricting, prothrombotic counterparts. Under different pathological conditions however, this balance can shift and the vasoconstrictive actions of thromboxane A_2 and PGH_2 can predominate. The two

isoforms of PGHS, PGHS-1 and PGHS-2, both play an important role in the regulation of this balance. Accordingly, in spite of their structural and functional similarity, they exhibit substantial differences in expression and activity. Whereas PGHS-1 is considered to be the constitutively expressed isoform regulating vascular tone, PGHS-2 is induced by inflammatory stimuli such as cytokines. PGHS-2 expression is associated with several cardiovascular risk factors and the development of atherosclerosis. [53, 54]

While both PGHS isoforms require the presence of lipid peroxides or $ONOO^-$ for activation, [55] PGHS-2 becomes activated at 10 times lower concentrations of lipid peroxides than PGHS-1. Consequently, independent activation of both isoenzymes is possible within one cell. Moreover, PGHS-2 displays a higher specific activity at lower arachidonate concentrations compared with PGHS-1, which also contributes to the differential activation of both enzymes. [56]

Interactions between •NO and prostaglandin H synthase—The first observation of an impact of •NO and NO_x on prostaglandin biosynthesis was made 15 years ago when it was shown that LPS increased the production of •NO and prostaglandins by murine macrophages and that inhibition of NOS decreased rates and extents of prostaglandin production. [57] Moreover, LPS-induced formation of prostaglandins was blunted by inhibition of NOS in LPS-treated mice and conversely, •NO donor administration increased prostaglandin synthesis. [58] Subsequent biochemical studies of purified PGHS-1 however, did not support that •NO mediated either the activation or inhibition of PGHS-1. [59, 60] Rather, the reaction product of •NO and $O_2•^-$, $ONOO^-$, was shown to activate both isoforms of PGHS. [55, 60] The mechanism proposed for this activation is similar to that appreciated for the PGHS "priming" function of lipid peroxides, wherein the formation of a Fe^{IV}=O radical (compound I) which is associated with a porphyrin pi cation radical or a nearby tyrosine or tryptophan radical and the subsequent intramolecular formation of a •Tyr-radical is initiated by the reaction of $ONOO^-$ with the Fe^{III}-heme of PGHS. [55, 61] Recent investigations have demonstrated that activation of PGHS by this mechanism is triggered by nanomolar levels of endogenous $ONOO^-$.[62]

Subsequent investigation revealed additional mechanisms whereby $ONOO^-$ reacts with PGHS. Purified PGHS-1 preparations and vascular smooth muscle cell studies revealed that $ONOO^-$ induces the nitration of Tyr385 of PGHS-1, leading to enzyme inactivation. [63] Nitrated PGHS-1 has been detected in human carotid endarterectomy samples and in atherosclerotic lesions of $ApoE^{-/-}$ mice, and is not detectable in $ApoE^{-/-}/iNOS^{-/-}$ mice. [63, 64] These contradictory observations of both PGHS activation and inhibition by $ONOO^-$ can be clarified by considering experimental conditions. The activation of PGHS-1 by $ONOO^-$ was only observed in the presence of a peroxide scavenger (glutathione peroxidase) suggesting that, in the absence of peroxides, $ONOO^-$ stimulates catalytic activity of PGHS-1 by oxidation of the Fe^{III}-heme [60] and thus provides the "peroxide tone" necessary for the activation of PGHS-1. [65] In the absence of a peroxide scavenger and in the inflammatory milieu of arteriosclerotic lesions, $ONOO^-$ can also inhibit PGHS-1 by nitration of Tyr385. These findings are also supported by a recent study demonstrating that the effect of $ONOO^-$ on PGHS is dependent on whether the enzyme is in the resting or active state. [29]

S-nitrosation is another mechanism whereby •NO can modulate PGHS activity, an event, shown to be facilitated by selective binding of iNOS to PGHS-2 both *in vitro* and in macrophages. This leads to S-nitrosation of Cys526 of PGHS-2 and enzyme activation, [66] an event only observed for PGHS-2. [60, 66] In physiologic concentrations NO_2^- is also able to inhibit PGHS-2 in the presence of peroxides via heme-catalyzed oxidation to $^•NO_2$ and nitration of a tyrosine residue.[67]

Another impact of •NO on PGHS-2 but not PGHS-1 is the upregulation of PGHS-2 protein synthesis at both the post-transcriptional and post-translational level via •NO-dependent alterations in MAPK signaling. [68–70] In contrast, •NO also induces tyrosine nitration of PGHS-2 and inhibits its activity. [71] Thus, PGE_2 production by fibroblasts from PGHS1-deficient mice is inhibited by •NO, while the same concentrations of •NO lead to an increase in PGE_2 production in fibroblasts of PGHS-2-deficient mice. [72] Moreover, in murine lung-derived endothelial cells iNOS-dependent •NO generation increased prostaglandin production, whereas cell exposure to higher •NO concentrations inhibited prostaglandin biosynthesis. [73] In aggregate, there is a differential regulation of PGHS-1 and PGHS-2 by •NO and secondary NO_x species, events that will be dictated by mediator concentrations and the underlying redox state of cells.

Nitric oxide and prostaglandin I_2 synthase—Another interaction between •NO and prostaglandin signaling occurs downstream of PGHS, the inhibition of prostaglandin I_2 synthase (PGI_2S) by $ONOO^{-}$. [74] The nitration of Tyr430 is induced by $ONOO^{-}$, inhibiting substrate access to the catalytic site of PGI_2S. [65, 75] Nitrated PGI_2S has been detected in diverse animal models of disease, including diabetes, [76] ischemia-reperfusion, [77] nitroglycerin tolerance, [78] septic shock [79] and atherosclerosis. [80] Since prostaglandin I_2 is a key vasodilatory prostanoid, its balance with platelet-derived thromboxane A_2 is critical for maintaining vascular homeostasis. Consequently, the inhibition of PGI_2S by •NO-derived species can decrease both rates of PGI_2 formation and PGI_2 concentration, resulting in the accumulation of PGH_2 and activation of the thromboxane A_2 receptor. [81] This underscores the critical cardiovascular actions of $ONOO^{-}$ where, by affecting PGI_2S activity, there can be a shift in the balance of vasorelaxant and vasoconstrictive prostanoids.[82]

Nitric oxide and lipoxygenases

Lipoxygenases (LOX) are non-heme iron-containing enzymes that catalyze the oxygenation of polyunsaturated fatty acids to unstable hydroperoxides, a process termed lipoxygenation. These hydroperoxides are rapidly reduced and converted to secondary, more stable signaling mediators. [83, 84] During lipoxygenation, fatty acid radical intermediates are formed, which usually remain enzyme-bound, but under pathological conditions can also be released from the enzyme to induce further free radical reactions. [85] Multiple LOX isoforms are expressed by mammals, which were initially named according to the carbon atom number of arachidonic acid where they introduce oxygen. A phylogenetic nomenclature has been suggested recently, [86] that allows for species-related differences. Also, LOX isoforms can introduce oxygen into both shorter and longer chain unsaturated fatty acids, can oxidize both free and esterified fatty acids and can oxygenate fatty acids at more than one position. [87–89] In humans, 5-LOX (also 5-LOX in the phylogenetic nomenclature) and 15-LOX (12/15LOX, new nomenclature) are expressed in reticulocytes and, upon activation, in monocytes.

There is a significant contribution of LOX-derived species to the pathogenesis of vascular inflammatory diseases, [84] with 5-LOX being the best characterized isoenzyme. 5-LOX reaction with arachidonic acid yields 5-hydroperoxyeicosatetraenoic acid (5-HPETE), an intermediate in the biosynthesis of the pro-inflammatory signaling mediators leukotrienes. 5-LOX is abundantly expressed in both, rodent models of atherosclerotic disease and in atherosclerotic lesions of humans, [90, 91] where it can generate chemotactic products that induce inflammatory cell accumulation [92] and exert direct vasoconstrictor actions. [93, 94]

15-LOX has been detected in monocytes, macrophages and platelets, with its expression induced by interleukin-4 and -13 with concomitant down-regulation of expression of 5-LOX. [91] Compared with 5-LOX, the physiological actions of 15-LOX are less apparent, although a role in antagonizing inflammatory responses has also been suggested. [95, 96]

The contributions of 15-LOX-derived species to inflammatory vascular diseases is complicated by contradictory data. On the one hand, rodent models of atherosclerosis (15-LOX knock out and 15-LOX overexpressing mice) suggest a pro-atherogenic role of 15-LOX. [97–99] This is confirmed by the detection of a spectrum of regioisomers of fatty acid oxidation products in clinical specimens of early atherosclerotic lesions that are indicative of LOX-catalyzed reactions, rather than more random free radical events. [83, 85] On the other hand rabbits overexpressing 15-LOX exhibited a marked decrease in atherosclerotic lesion area. [100, 101] The reasons for these contradictory findings remain elusive, with concentration-dependent signaling responses to the HPETE products of 15-LOX suggested as an explanation. [83]

LOX activation inhibits •NO signaling and conversely, •NO inhibits LOX enzymatic activity and product formation. [49, 102] The LOX-dependent oxidation of polyunsaturated fatty acids requires oxidation of the non-heme iron from the ferrous (Fe_2^+) to the ferric state (Fe_3^+). It was first proposed that •NO forms an inhibitory iron-nitrosyl complex with the enzyme in its ferrous form. [23, 103] This reaction requires very high and non-physiological concentrations of •NO to detect the iron-nitrosyl complex however, with LOX inhibition by •NO detectable at much lower concentrations. [102, 104] Current data support that •NO reacts with LOX-bound lipid peroxyl radicals to yield an organic peroxynitrite (LOONO) intermediate. This mechanism of inhibition is kinetically rapid (Figure 1), was observed to occur at physiological concentrations of •NO (nM) and ultimately yields a fatty acid hydroperoxide and nitrite as products. After the hydroperoxide is released by LOX however, the enzyme is left in an inactive ferrous form, requiring reactivation for another catalytic cycle. [49] These reactions with •NO have been defined for 15-LOX, but because of similar catalytic mechanisms of other LOX isoenzymes it can be assumed that these events are also applicable to 5-LOX. Accordingly, •NO-dependent suppression of 5-LOX metabolism has been shown in cell and animal models. [105, 106] The inhibitory effects of •NO and $ONOO^-$ on 5-LOX has also been associated with tyrosine nitration and S-nitrosylation of 5-LOX. [107, 108] These latter data stem from isolated enzyme and cell studies, with confirmatory studies in animal models remaining to be done.

Nitric oxide and cytochrome P 450 (CYP) enzymes

CYP enzymes are membrane-bound, heme-containing oxidases best known for catalyzing the disposition of xenobiotics. While most CYP enzymes are predominantly expressed in the liver, many CYP isoenzymes are present in extrahepatic locations such as endothelial and vascular smooth muscle cells and contribute to cardiovascular regulation. [109, 110] In this context the CYP-2 family of enzymes having epoxygenase activity and the CYP-4 family, which consists of ω-hydroxylases, are of particular significance.

The epoxygenases of the CYP-2 family catalyze the generation of different epoxide regioisomers from arachidonic acid, termed epoxyeicosatrienoic acids (EETs). Because of an ability to hyperpolarize endothelial and smooth muscle cells and mediate vasodilatation independently of •NO and PGI_2, EETs are one component of a collection of species given the functional terminology endothelium-derived hyperpolarizing factor (EDHF). [110–112] The CYP-4 family, specifically the CYP-4A family, generates an essential mediator of angiotensin II and endothelin-1 signal transduction, 20-hydroxyeicosatetraenoic acid (20-HETE). [113, 114]

Substantial evidence supports that •NO inhibits CYP isoenzymes, [115, 116] with different mechanisms proposed to account for this inhibition. First, CYP inhibition induced by formation of a reversible heme iron-nitrosyl-complex has been suggested [117] and supported by observations in a rat model exposed to LPS-induced, iNOS-derived •NO. [118] For some isoforms, CYP inactivation by $ONOO^-$-induced tyrosine nitration has been reported, but the physiological relevance of these findings remain to be elucidated. [119, 120] Moreover, it was suggested that •NO also induces proteasomal degradation of CYP protein [121] and affects CYP transcription [122].

Overall, the cardiovascular impact of CYP inhibition remains to be defined due to contradictory observations. For example, the inhibition of HETE synthesis enhances the vasodilatory effects of •NO. [123] Alternatively, decreased •NO bioavailability enhances CYP-dependent synthesis of EET and thus serves as a "reserve" vasodilatory mechanism. [124, 125] Since •NO can impact both the synthesis of EET (CYP 2) and HETE (CYP-4A), [123, 126] these findings are in conflict and suggest a more complex interaction between •NO and CYP-derived lipid mediator formation and action. Similarly, studies assessing the effect of CYP-2C9 on acetylcholine-induced vasodilation of the forearm report conflicting results. [127, 128]

CYP-derived fatty acid oxidation products also influence the bioavailability of •NO. For example, exposure of vascular endothelial cells to 5,6-EET stimulates •NO synthesis by increasing intracellular Ca^{2+} levels. [129] In contrast, CYPs can also accelerate the vascular generation of ROS. Specifically, porcine artery CYP-2C serves as a source of ROS that react with •NO and impair •NO-dependent vascular relaxation. [124] Recent findings also suggest an impact of 20-HETE on •NO-biosynthesis by uncoupling eNOS, enhancing eNOS-derived ROS production and the subsequent inhibition of •NO signaling. [130] Again, how these paradoxical findings can be integrated into the physiology of vascular function remains to be clarified in vessel and organ preparations and in in vivo models.

Nitric Oxide and Redox-Derived Lipid Mediators

Redox-derived metabolites of •NO

Nitric oxide exerts a pervasive influence on the generation and actions of lipid signaling mediators, by modulating both enzymatic- and free radical/oxidant-dependent unsaturated fatty acid oxygenation. When lipid oxidation is initiated by free radical/oxidant species, •NO and its byproducts can either inhibit or stimulate fatty acid oxidation, [131] yielding products that can display unique signaling actions. The net biochemical and physiological outcome when reactions of •NO and oxidizing lipids converge will be dictated by multiple conditions, including the specific composition and concentrations of the oxidative inflammatory milieu. This includes free radical and oxidizing species (e.g., $O_2•^-$, H_2O_2, •OH, $ONOO^-$, $•NO_2$ HOCl, metal catalysts), oxidant scavengers (e.g., tocopherols, glutathione, thioredoxin, catalase, superoxide dismutases, peroxidases), and other aspects of the local chemical milieu (e.g., pH, availability of fatty acids, relative lipophilicity). Oxidized lipid mediators not derived from PGHS, LOX and CYP450 typically stem from autocatalytic lipid peroxidation reactions occurring in membranes and lipoproteins. The kinetically favourable reaction of $O_2•^-$ with •NO in particular gives a unique initiator of lipid peroxidation, $ONOO^-$. Notably, $ONOO^-$-induced lipid oxidation proceeds without metal catalysis, in contrast to H_2O_2. [131, 132] Moreover, since •NO is a radical itself, it readily reacts with lipid peroxyl radicals and can rapidly terminate chain propagation reactions of lipid oxidation. Thus, the extent and product profile of $O_2•^-$ induced lipid oxidation will be a function of relative concentrations of •NO. The interactions of •NO with non-enzymatically generated lipid mediators are exemplified in the studies outlined below (see also Figures 1 and 2 for an overview of interactions between •NO and lipid mediators).

Nitric oxide and isoprostanes

Isoprostanes are prostaglandin-like compounds formed during free radical catalyzed auto-oxidation of polyunsaturated fatty acids that proceed independently of PGHS. [133] Isoprostanes are generated at increased rates during inflammatory states. Notably, iNOS-derived •NO contributes significantly to the formation of isoprostanes, with $iNOS^{-/-}$ mice displaying reduced levels of isoprostane formation. [134, 135] In support of a role for $ONOO^-$ as the proximal mediator of fatty acid oxidation to isoprostane derivatives, $ONOO^-$ will directly induce formation of F_2-isoprostanes during the oxidation of lipoproteins and plasma lipids. [136]

Consistent with its ability to terminate chain propagation reactions of lipid oxidation, •NO, when present in excess of O_2•$^-$, can also inhibit oxidant-induced F_2-isoprostane formation. [137] Of note, there is an inverse correlation between net extents of •NO synthesis and free radical-induced 15-F_{2t}-isoprostane production in subjects with type 1 diabetes, an event not observed in control subjects. This is consistent with the ability of •NO, under these conditions, to inhibit lipid peroxidation. [138]

Several groups of isoprostanes are found in vivo, with F_2-isoprostanes the most abundant. As in the case of prostaglandins the letter F indicates the type of cyclopentane ring, thus F_2-isoprostanes share structural similarity with prostaglandin $F_{2\alpha}$. Correspondingly, D_2, E_2, G_2, and H_2 isoprostanes have been described, which are isomers of their respective prostaglandin. Each class of isoprostanes contains 64 isomers, made up of four regioisomers each and consisting of eight racemic diastereoisomers. [139] In addition to these isoprostane classes, two other groups have been described, the A_2- and J_2-isoprostanes, which have an electrophilic cyclopentenone ring instead of the cyclopentane ring, a property that influences their mode of signaling.

F_2-isoprostanes are extremely robust biomarkers of oxidative stress-induced lipid peroxidation. [139–141] In vivo generation of F_2-isoprostanes is most commonly assessed by measurement of the urinary metabolite of 15-F_{2t}-isoprostane, 2,3-dinor-5,6-dihydro-15-F_{2t}-isoprostane. [139, 142] However, F_2-isoprostane derivatives can also be detected in plasma and measurement of esterified isoprostanes in plasma lipoproteins or tissue biopsy samples is also an option. [142, 143]

Most studies concerning the signaling actions of isoprostanes have investigated F_2-isoprostanes, especially 15-F_{2t}-isoprostanes. In animal models, 15-F_{2t}-isoprostanes are potent vasoconstrictors in vascular beds including renal arteries, [144] pulmonary arteries, [133] coronary arteries, [145] and cerebral arterioles. [146] Moreover, an involvement in obstructive pulmonary disease has been suggested. [147] Similarly, vasoconstriction of pulmonary [148] and renal arteries [149] as well as airway constriction [148] has been found for E_2-isoprostanes, in particular 15-E_{2t}-isoprostanes. In addition to these excitatory effects on smooth muscle cells, F_2-isoprostanes induce platelet activation, [150] increase endothelin-1 expression [151] in endothelial cells and are generated by oxidizing LDL. [152] Accordingly, a role may exist for isoprostanes in the pathophysiology of atherosclerosis, with 15-F_{2t}-isoprostanes detected at increased levels in ApoE and LDL-receptor-deficient mice. [153]

The signaling actions of E_2-and F_2-isoprostanes are viewed to be receptor-mediated, however the specific receptors involved remain to be clarified clinically. Agonism of the thromboxane A_2 receptor is proposed to be responsible for the vasoconstrictive and proatherogenic effects of F_2-isoprostanes, from studies of transgenic mice. [154, 155] In contrast, in platelets F_2-isoprostanes have been shown to act as an antagonist of this receptor. [156] While isoprostanes are also proposed to serve as peroxisome proliferator-activating receptor γ (PPARγ) ligands, [157] the evidence for a unique isoprostane receptor is limited to in vitro studies. [155, 158]

Conversely, signal transduction by A_2- and J_2-isoprostanes is not ascribed to specific ligand-receptor interactions. Due to the α,β-unsaturated carbonyl group of the cyclopentenone ring, these compounds exhibit an electrophilic reactivity which enables a Michael addition with nucleophilic residues, typically thiol-containing biomolecules which in the case of proteins can result in an altered function of these proteins. [159] This type of posttranslational modification by electrophiles is emerging as a significant pathway of signal transduction, with the signaling actions of these derivatives differing substantially from the aforementioned isoprostane classes. Thus, 15-A_2- and 15-J_2-isoprostanes exhibit anti-inflammatory properties, such as inhibition of NF-κB-dependent expression of iNOS and $PGHS_2$ in LPS-stimulated murine macrophages.

[160] Overall, the signaling actions of isoprostanes and the impact of •NO appear to be tissue, regioisomer and species specific, thus extrapolation of individually-reported effects should be made with caution (see [139] for a recent, more detailed review on the biological effects of isoprostanes).

Nitric oxide and reactive aldehydes

Non-enzymatic oxidation of polyunsaturated fatty acids yields a variety of reactive aldehydes, which are derived from lipid hydroperoxides undergoing acyl chain cleavage. These products of lipid peroxidation are also sometimes useful markers of oxidative inflammatory conditions and include species such as malondialdehyde (MDA), glyoxal, acrolein, 4-hydroxy-2-nonenal (4-HNE) and 4-oxo-2-nonenal (ONE). While these byproducts have traditionally been regarded as cytotoxins, they are also increasingly recognized to manifest cell signaling actions.

The best characterized lipid aldehyde derivative is HNE, which contains an α,β-unsaturated carbonyl group and thus displays electrophilic reactivity towards thiols, histidine and other biological nucleophiles. At high concentrations, this reactivity can support cytotoxic reactions with DNA bases and other cellular components, while in lower concentrations α,β-unsaturated carbonyls can induce the posttranslational modification of a wide variety of proteins and indirectly act as anti-inflammatory signaling mediators.

For example, HNE induces a broad range of actions on protein kinase signaling by activating multiple isoforms of protein kinase C, [161, 162] several members of the MAP kinase family, [163, 164] and tyrosine kinase receptors, [165] as well as inhibiting most catalytic thiol-containing protein tyrosine phosphatases. [166, 167] HNE also inhibits mitochondrial aldehyde dehydrogenase-2 (ALDH-2) leading to cardioprotection in a rodent model of ischemia. [168]

In terms of regulating transcription factor function, HNE inhibits NF-κB signaling by preventing the phosphorylation of IκB [169] and activates nuclear factor E2-related transcription factor (Nrf2)-regulated gene expression, [170] leading to increased expression of the antioxidant actions of heme oxygenase-1 (HO-1), peroxiredoxin and stress protein A 170.

As noted, •NO and •NO-derived reactive species can initiate and propagate membrane lipid peroxidation, and even inhibit this process when •NO is in “excess” of inciting oxidants. [51, 102] Hence, increased generation of reactive aldehyde products of lipid oxidation are to be expected in inflammatory states involving enhanced generation of ROS and elevated •NO concentrations as a consequence of increased iNOS expression. Indeed, it was shown in insulin-secreting RINm5F cells that •NO donors, as well as interleukin-1β induced iNOS expression leads to increased production of MDA and 4-HNE. This was inhibited by manganese superoxide dismutase (Mn-SOD), suggesting an involvement of $ONOO^-$ in stimulating lipid oxidation. [171] Furthermore, in a rat model of renal ischemia-reperfusion (I/R), post-I/R infusion of L-arginine into kidneys increased organ production of 4-HNE. [172] Similarly, in a model of DOCA-salt-induced hypertension and cardiac hypertrophy, $iNOS^{-/-}$mice exhibited a significantly reduced myocardial production of 4-HNE, in comparison to wild-type mouse myocardium. [173] Finally, there is a contribution of iNOS in lipid radical generation and the formation of 4-HNE in streptozotocin-induced diabetes. [174]

There is an opposing influence of 4-HNE on •NO-homeostasis. This is evidenced by a dose-dependent HNE inhibition of LPS/interferon-γ induced •NO production and iNOS-expression in vascular smooth muscle cells, an effect mediated by HNE inhibition of NF-κB signaling. [175] Moreover, it has been suggested that 4-HNE also reduces •NO production by modulation of methylarginine levels and DDAH activity. For example, bovine aortic endothelial cell exposure to 4-HNE decreases DDAH catalytic activity. In this model system, DDAH-overexpression and L-arginine supplementation partially reversed the inhibition of •NO

production. [176] This multifaceted inhibition of •NO production can significantly influence the progression of inflammatory conditions. [177] Thus, the lipid oxidation product 4-HNE might serve as a negative feedback mechanism to counteract overt production of •NO by iNOS. How these findings are applicable in vivo to cardiovascular events remains to be defined.

Nitric oxide and fatty acid nitration

Nitro-fatty acids (NO_2-FA) are endogenous signaling mediators, which exhibit anti-inflammatory signaling actions. NO_2-FA are generated from the reaction of •NO-derived species with unsaturated fatty acids and constitute a biochemical convergence of reactants participating in •NO and lipid signaling. During peroxyl radical-induced fatty acid chain propagation reactions, NO_2-FA emerge as termination products in the presence of •NO and its oxidative byproducts. [30, 102, 131, 178] Different biochemical mechanisms, reviewed elsewhere, [179] account for the generation of NO_2-FA, with all having $•NO_2$ in common as the proximal nitrating species. Basal plasma and tissue levels of NO_2-FA are in the nM concentration range, with inflammatory events such as ischemia-reoxygenation leading to higher concentrations in specific compartments such as mitochondria or macrophages. [180–183]

As for cyclopentenone isoprostanes and reactive aldehydes, the electrophilic adduction of biological targets is central to how NO_2-FA transduce signaling actions. The robust electrophilic reactivity of NO_2-FA is explained by the high electronegativity of the NO_2 group, especially when bound to an olefinic carbon of unsaturated fatty acids. This facilitates Michael addition reaction between the alkenyl β-carbon of NO_2-FA and nucleophiles such as protein histidine and cysteine residues, a process which in the case of NO_2-FA is termed nitroalkylation. [184] By virtue of this reactivity, NO_2-FA inhibit pro-inflammatory cytokine secretion in LPS-stimulated macrophages by repressing NF-κB dependent target gene expression, an effect which is induced by the nitroalkylation of functionally critical cysteines in the p65 subunit of NF-κB. [185] In addition, NO_2-FA inhibit vascular smooth muscle cell proliferation by activation of Keap1/Nrf2-(Kelch-like ECH-associating protein 1/nuclear factor erythroid 2-related factor 2)-mediated signaling. Nitroalkylation of the cysteine-rich cytoplasmatic suppressor protein Keap1 leads to dissociation of Keap1 from the transcription factor Nrf2, allowing translocation of Nrf2 to the nucleus. [186] Another significant signaling action of NO_2-FA is the ability to activate PPARγ and to a lesser extent PPARα and PPARδ. [187] NO_2-FA have a receptor affinity exceeding virtually all putative endogenous PPARγ ligands, and that rivals the receptor affinity of synthetic thiazolidinedione ligands (e.g., Rosiglitazone). Similar to thiazolidinediones NO_2-FA induce increased glucose uptake in adipocytes. [188] Of note, NO_2-FA exhibit a reduced potential to induce adipogenesis and adipocyte triglyceride accumulation in comparison to rosiglitazone, a property currently viewed to be a consequence of the distinctive PPAR-coregulator protein interactions induced by NO_2-FA.[179, 187]

Besides signal transduction reactions, which depend on electrophilic reactivity, NO_2-FA signal via several other mechanisms. While not viewed to be clinically significant, NO_2-FA release •NO and thus under certain circumstances might serve as reservoir for •NO. NO_2-FA-dependent •NO release is mediated by a Nef-like reaction, involving the aqueous conversion of nitro compounds into carbonyls. The resulting radical is stabilized by conjugation with the alkene and by the hydroxy-group, a moiety known to stabilize radicals. [189, 190] As a consequence, NO_2-FA mediate the cGMP-dependent relaxation of preconstricted rat aortic vessel rings. [191] The occurrence of this reaction in vivo however, awaits definitive confirmation, since NO_2-FA are resistant to aqueous-mediated decay reactions when stabilized by membrane and lipoprotein lipids or upon protein adduction. [179] Other anti-inflammatory signaling actions of NO_2-FA include inhibition of neutrophil and platelet activation via non-cGMP dependent mechanisms. Thus, nitrolinoleic acid (LNO_2) inhibited $O_2^{•-}$ production,

azurophilic degranulation, calcium mobilization and CD 11b expression in activated polymorphonuclear neutrophils. [192, 193] Moreover, in platelets LNO_2 inhibited thrombin-induced aggregation. [193] For both cell types it was suggested that these mechanisms are mediated by an induction of adenylate cyclase activity. A final mechanism whereby NO_2-FA mediate anti-inflammatory signaling is via upregulation of endothelial and epithelial HO-1 gene expression. [194, 195] Induction of HO-1 expression by NO_2-FA is independent of •NO, NF-κB and PPAR-regulated mechanisms. Instead, transcriptional regulation by the Keap1/Nrf2 and the cAMP-dependent response element has been proposed. [194] HO-1 upregulation is a central protective response during many inflammatory processes including sepsis, arteriosclerosis and transplant rejection. A recent study showed generation of an endogenous NO_2-FA in Langendorff rat hearts subjected to ischemic preconditioning. Furthermore experiments on isolated cardiomyocytes subjected to anoxia/reoxygenation performed in this study suggest cardioprotective effects of these compounds. [183] Studies investigating the cardioprotective and anti-atherosclerotic actions of these compounds in animal studies are currently ongoing.

Conclusions

Nitric oxide and lipid signaling converge via multiple mechanisms, with the eventual biological outcome from these interactions depending to a great extent on the chemical equilibria of all potential reactants in a particular biological locus. This presents significant challenges in translating in vitro-based observations to more clinically relevant conditions. Multiple analytical and conceptual challenges also remain, as investigators strive to gain a detailed understanding of the myriad of signaling reactions mediated by the family of redox signaling mediators stemming from •NO. This includes the redox-related •NO byproduct $ONOO^-$, partially reduced oxygen species (e.g., $O_2•^-$, H_2O_2 and •OH), oxidized halides (e.g., HOCl, HOBr), oxidized thiols, S-nitrosothiols and oxidized or nitrated fatty acids. Due to the broad reactivity of •NO and kinetic considerations, the individual byproducts discussed in this review are never formed exclusively, and they often display overlapping reactivities with target molecules. A case in point is when cells or tissues are exposed in vitro to reactive mediator concentrations not experienced in vivo. For example, a cell faced with a high concentration of •NO coming from external addition of •NO donors or transfection of NOS would be expected to undergo thiol S-nitrosation, the oxidation of thiols to disulfides and higher oxidation states and the formation of mixed disulfides with glutathione. Additionally, $ONOO^-$ formation, fatty acid oxidation or nitration and FeS complex oxidation would be expected. As the field of redox signaling moves forward, it remains imperative that we continue to incisively define not only discrete mechanisms of redox signaling, but also that we strive to translate these reactions into an understanding of how they impact cell, vascular and organ function. As we gain additional insight from both in vivo models and clinical measurement of the molecular events and physiological responses to redox mediators, we will also be better positioned for developing new drug strategies.

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Non-standard abbreviations and acronyms

CYP	cytochrome P
DDAH	dimethylarginine dimethylaminohydrolase
5-HPETE	5-hydroperoxyeicosatetraenoic acid

NOS	NO synthase
EET	epoxyeicosatrienoic acids
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial NO sythase
iNOS	inducible NO synthase
nNOS	neuronal NO synthase
sGC	guanylate cyclase
cGMP	cyclic guanosine monophosphate
MAPK	mitogen-activated protein kinase
NFκB	nuclear factor κB
PG	prostaglandin
PGHS	prostaglandin H synthase
PGI_2S	prostaglandin I_2 synthase
PPAR	peroxisome proliferator-activating receptor
ROS	reactive oxygen species
RNS	reactive nitrogen species
LPS	lipopolysaccharids
LOX	lipoxygenases
MDA	malondialdehyde
4-HNE	4-hydroxy-2-nonenal
ONE	4-oxo-2-nonenal
HO-1	heme oxygenase-1
NO_2-FA	nitro-fatty acids
Keap1	Kelch-like ECH-associating protein 1
Nrf2	nuclear factor erythroid 2-related factor 2
LNO_2	9, 10, 12 and 13-nitro-linoleic acid

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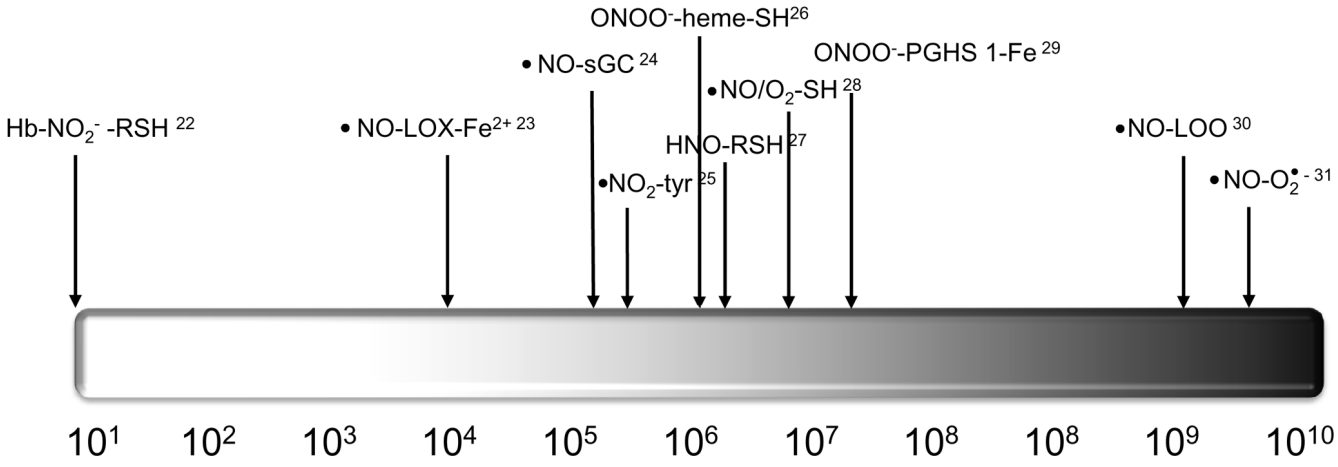

Figure 1. Reaction rate constants for critical reactions of •NO and •NO-derived species with key molecular targets that transduce cell signaling events

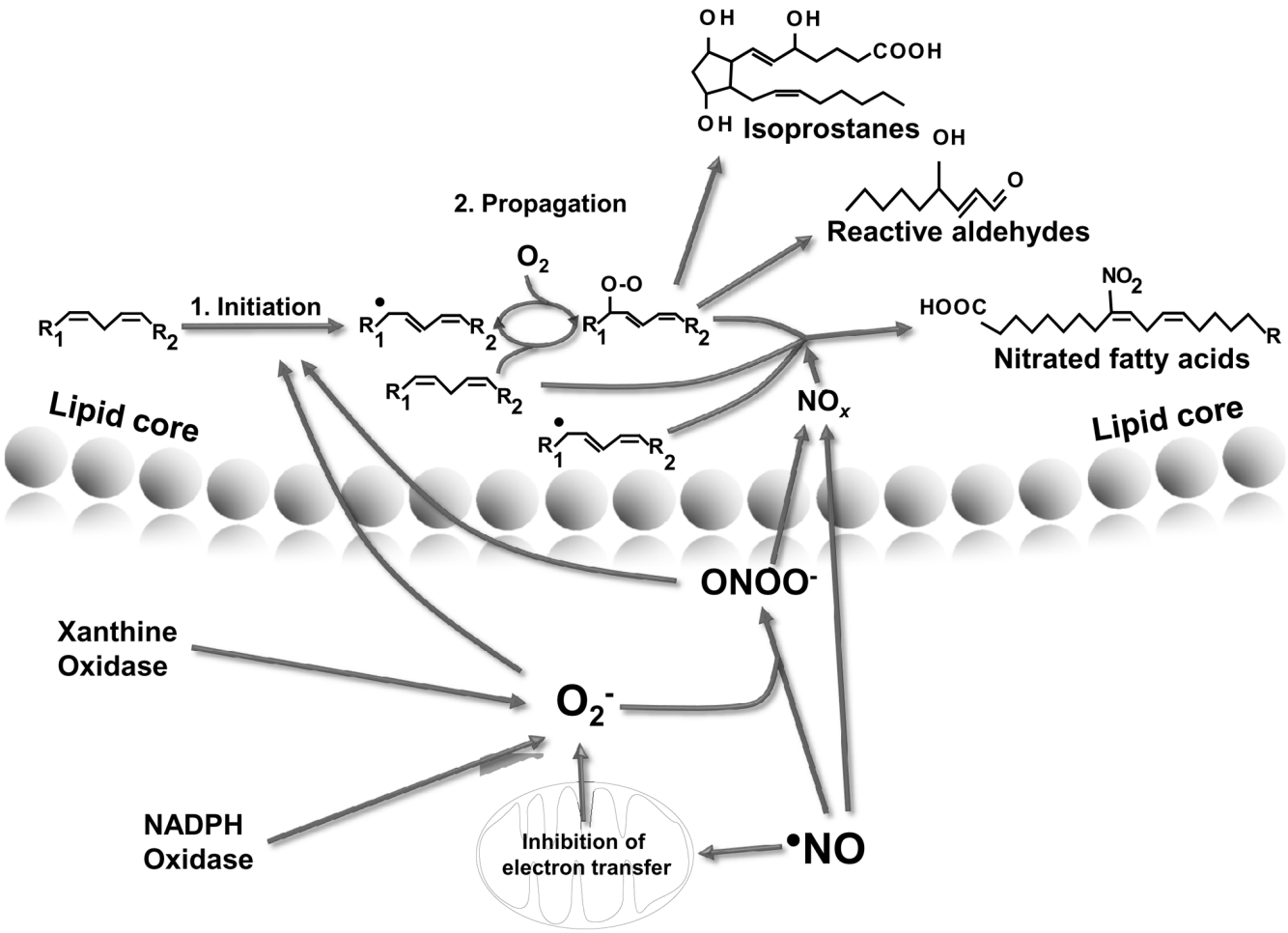

Figure 2. Formation of lipid signaling mediators from redox reactions within the lipid core of lipoproteins or cell membranes

(1) Oxidation of membrane lipids by free radicals and oxidants initiate lipid peroxidation, by formation of lipid radical species that then react with molecular oxygen or other radicals to form lipid peroxyl radicals. Peroxyl radical species can then either further propagate lipid peroxidation events or they can (2) serve as precursors for the generation of isoprostanes and reactive aldehyde derivatives. Finally, both native fatty acids or oxidized fatty acids can react with nitrogen dioxide ($\bullet NO_2$) arising from secondary reactions of •NO and nitrite (NO_2^-) leading to the formation of nitro-fatty acid derivatives (3)