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ELECTROPHILIC NITRO-FATTY ACIDS: ANTI-INFLAMMATORY MEDIATORS IN THE VASCULAR COMPARTMENT

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Abstract

Vascular inflammatory disorders are often associated with both decreased NO bioavailability and a lack of responsiveness to NO, a consequence of impaired NO biosynthesis, dysregulated L-arginine metabolism, endothelial nitric oxide synthase (eNOS) uncoupling and NO consumption induced by redox reactions of NO. The latter is mediated via oxidative inflammatory conditions altering NO-dependent endothelial function, including vascular tone and cell proliferation. The redox reactions of NO and byproducts such as nitrite can react to yield electrophilic nitro-fatty acid derivatives (NO_2-FA) and exemplifies a biochemical convergence of reactions participating in NO and lipid signaling. NO_2-FAs represent a novel therapeutic strategy to treat vascular disorders by improving endothelial dysfunction through enhancing NO signaling and blocking vascular smooth muscle proliferation, inflammation, and maladaptive remodeling.

Introduction

Endothelial dysfunction is a hallmark of vascular inflammatory disorders and plays a central role in mediating structural changes such as lipid accumulation or intimal hyperplasia in the vasculature. Endothelial NO plays a critical role in the regulation of vascular homeostasis by inhibiting inflammatory cell function and smooth muscle proliferation [1,2]. Oxidative inflammatory conditions, through NO- and O_2-derived species, results in oxidative stress, decreased antioxidants, and lower NO bioavailability. This in turn incites a vicious cycle of endothelial dysfunction, vascular cell proliferation and vascular remodeling (Figure 1) increasing susceptibility to atherosclerosis, hypertension, thrombosis, and diabetes mellitus. For example, drugs that make soluble guanylate cyclase (sGC) more responsive to NO and that increase cellular cGMP levels can protect hypoxic mice from developing pulmonary artery hypertension (PAH), but knockout mice lacking eNOS fail to respond as they are incapable of endothelial NO generation [3]. Increasing NO signaling can partially reverse PAH and pulmonary vessel remodeling once PAH has been established [4••,5••]. Moreover, statins have anti-atherogenic effects mediated, in part, through scavenging superoxide, increasing eNOS expression and NO production, and upregulation of heme oxygenase 1 (HO-1) expression [6, 7]. Since vascular inflammatory disorders are associated with endothelial dysfunction and impaired NO function, the targeting of various aspects of the NO signaling pathway has been

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proposed as a therapeutic modality. We overview the potential impact of NO_2-FA on modulating endothelial gene expression and function as a therapeutic strategy, with these species exhibiting anti-inflammatory cell signaling properties that upregulate HO-1, eNOS and NO production in the vasculature, inhibit VSMC proliferation, and activate PPARγ through Nrf2-dependent and independent processes. Moreover, we address that all of these signaling actions are attributable to the electrophilic nature of NO_2-FA.

Formation of NO_2-FAs

Both NO- and nitrite (NO_2^-)-derived species yield the nitrating products that mediate the nitration of unsaturated fatty acids [8,9]. When it was observed that the reaction product of NO and $O_2^{\cdot -}$, peroxynitrite ($ONOO^-$), was a potent biological oxidizing and nitrating agent, the tissue formation and actions of 3-nitro-tyrosine became of interest [10]. The same reactions that nitrate tyrosine also yield NO_2-FA, with the prevalence and redundancy of these mechanisms supporting that nitration reactions in part act to transduce NO signaling and tissue inflammatory responses. Both free radical and ionic mechanisms that share nitrogen dioxide ($.NO_2$) as the proximal nitrating species have the ability to generate NO_2-FA [11]. NO also rapidly intercepts free and enzyme-bound lipid alkoxyl and peroxyl radicals ($k = 2 \times 10^9$ $M^{-1}s^{-1}$) [9,12] during both autocatalytic and enzymatic fatty acid oxygenation reactions, also yielding nitrated products [9,13,14]. Moreover, metabolic, inflammatory and acidic conditions promote fatty acid nitration [15,16]. For example, cardiac tissue and mitochondrial fatty acid nitration is increased after ischemia-reperfusion (I/R) reactions *in vivo* [17••]. Importantly, NO_2-FA are reversibly-reactive electrophiles that covalently adduct nucleophilic amino acids present in low molecular weight peptides and proteins and that rapidly undergo β-oxidation to shorter chain nitroalkenes and further metabolism to hydroxyl and keto derivatives [17••–19]. Overall, biological fatty acid oxidation and nitration reactions yield an array of NO_2-FA regioisomers that display unique chemical reactivities and signaling actions.

Mechanisms of Electrophilic Fatty Acid Reaction

Unsaturated fatty acids can be converted to electrophilic products via enzymatic and non-enzymatic oxidation and nitration reactions [20]. Electrophiles (“electron-lover”) undergo chemical reactions by attacking nucleophiles, accepting an electron pair and forming a chemical bond. Electrophilic species can also be ingested from the diet and are endogenously produced as metabolic byproducts of redox reactions. Some electrophiles permanently and irreversibly modify a target protein and others induce more short-lived and reversible adduction of the target protein [21]. NO_2-FAs are Michael acceptors that react with nucleophiles such as the cysteine thiolate, the imidazole moiety of histidine and the ε-amino group of lysine residues. These reactions facilitate the reversible adduction and post-translational modification of proteins to alter structure, trafficking and catalytic activity [20]. Reversibly-reactive electrophiles typically display low or no cytotoxic effects at low concentrations, with these reactions potentially functioning as signaling events that are sensitive to cellular metabolic and redox status. The reversible adduction of cysteine by electrophiles can include inter- or intramolecular exchange reactions between different thiols, with ultimate transfer of the electrophile to GSH and export of the electrophile-GSH adduct from cells through specific multi-drug resistance protein transport mechanisms [22] (Figure 2). This electrophile adduction of GSH can lead to a depletion of GSH pools, thereby altering the redox status of the cell and either directly or indirectly activating compensatory responses (Nrf2 activation) and inducing tissue-protective gene expression. Pharmacologic interventions directed towards activating an integrated system that senses, responds to and controls levels of electrophiles may lead to new strategies for drug discovery and treatment of inflammatory disorders.

Electrophile-induced Nrf2 activation

Electrophilic fatty acid derivatives mediate cytoprotective cell signaling reactions via phase 2 gene expression. Biological electrophilic fatty acids, which include NO_2-FA, cyclooxygenase-derived 15-deoxy-$\Delta^{12,14}$-PGJ_2, a variety of isoprostane derivatives and lipoxygenase-derived α,β-unsaturated ketones [23,24], are emerging as mediators protectiing against xenobiotic and oxidant injury. The transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2)/Keap1 (Kelch-like ECH-associating protein) pathway mediates phase 2 gene activation. Under normal conditions, Nrf2 localizes to the cytoplasmic supressor protein Keap1 which has several critical cysteine residues that serve as sensors to environmental stresses such as ROS and electrophiles. Keap1 cysteines are oxidized or alkylated, causing a conformational change and liberating Nrf2 to translocate to the nucleus, bind to the *cis*-acting DNA regulatory antioxidant response element [ARE, also referred to as the electrophile response element (EpRE)], and thereby transactivating Nrf2-dependent gene transcription. This includes enzymes of GSH synthesis and transfer, quinone reductase (NQO1), epoxide hydrolase, thioredoxin, transferrin, catalase, superoxide dismutase, and HO-1 [25]. This widespread mechanism, conserved in both plants and animals, protects against metabolic and inflammatory stress.

NO_2-FAs upregulate adaptive protective mediators

NO_2-FAs reversibly react with susceptible protein thiols of Keap1 and modulate phase 2 gene expression responses that attenuate vascular anti-inflammatory activity. NO_2-FAs induce HO-1, NQO1, and GSH biosynthetic enzyme (GCLM) expression by Nrf2-dependent processes. This induction of HO-1, GCLM and NQO1 mRNA and protein expression was significantly attenuated in cultured human endothelial cells transfected with Nrf2-siRNA [26]. This demonstrates that NO_2-FAs mediated induction of tissue-protective responses in endothelial cells is partially regulated by the Nrf2/Keap1 pathway.

NO_2-FAs Increase eNOS and HO-1 expression

NO_2-FA, as NO and NO_2^--derived species, can induce “feedback” regulation of eNOS expression and activity. Administration of OA-NO_2 (3 mg/kg/d for 3 d) to mice via a subcutaneously-implanted osmotic mini-pump resulted in an 8-fold increase in plasma OA-NO_2 levels compared to oleic acid (OA) controls. OA-NO_2 increased aortic eNOS and HO-1 mRNA expression 3-fold, as determined by multiplex real time PCR analysis. Cultured endothelial cells responded similarly to NO_2-FAs added to the culture medium, displaying increased eNOS and HO-1 mRNA and protein expression [27,28]. Additionally, OA-NO_2 increased the release of NO in endothelial cells [27]. Thus NO_2-FAs induce signaling reactions that increase eNOS–dependent NO production and HO-1 expression, both *in vitro* and *in vivo*.

NO_2-FAs inhibit VSMC proliferation

NO_2-FA inhibited serum-induced VSMC proliferation in a dose-dependent manner with native fatty acids having no effect. Analysis of cell-cycle protein expression revealed upregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ without affecting expression levels of cyclin D1 and E or cyclin-dependent kinase 4. Knock-down of Nrf2 using a si-RNA approach abolished NO_2-FA-mediated growth inhibition in VSMCs and the upregulation of $p27^{kip1}$ protein expression. Conversely, Ad.Nrf2 increased $p27^{kip1}$ and ectopic expression of Keap1 attenuated the upregulation of $p27^{kip1}$ by Ad.Nrf2 in a dose-dependent manner [29••]. These data support that NO_2-FAs inhibit VSMC proliferation and that this action is, in part, dependent on Nrf2 activation.

NO_2-FAs are partial PPAR agonists

Peroxisome proliferator-activating receptor (PPAR) agonists have been plagued by adverse side effects. Partial PPAR agonists that retain their efficacy without adverse side effects appear to be the next generation of signaling activators [30]. One such mechanism of partial PPAR activation focuses on differential recruitment of coactivators and/or corepressors to the receptor, resulting in a tissue-and promoter-selective expression of specific target genes. NO_2-FAs and keto-fatty acid derivatives have high binding affinities for all three PPAR isotypes, with PPARγ the most robustly-activated receptor (followed by α and then δ) [31, 32•]. Reporter cell transactivation studies revealed that different regioisomers of NO_2-FA behave as partial agonists [33•]. Receptor-ligand-binding analysis indicates that NO_2-FA covalently react with the ligand binding domain Cys285 residue of PPARγ in both biochemical reaction systems and cells. While saturation kinetics does not apply to this mode of receptor interaction, comparative studies support a greater EC_{50} for receptor activation than that of the synthetic PPARγ agonist Rosiglitazone (Rosi) [34•]. NO_2-FA transactivation of PPARγ is not induced by similar concentrations of non-electrophilic NO_2-FA metabolites, NO donors, native oleic or linoleic acid or oxidized derivatives of these fatty acids in the presence and absence of NO [31]. NO_2-FAs act as partial PPARγ agonists in nM ranges, unlike native fatty acid, prostaglandin metabolite and oxidized fatty acid derivatives that only activate PPAR α, γ and δ at non-physiological concentrations of >50 μM [35–37].

Inasmuch as endothelial dysfunction plays a vital role in both systemic and pulmonary vascular diseases [38–40], it is notable that PPARγ agonists increase NO production in EC by post-translational eNOS modifications and not by increased eNOS expression [41–43]. Moreover, endothelial PPARγ−/− mice were hypertensive (significantly higher mean arterial pressure), released less NO, and displayed increased NFkB-DNA binding compared to littermate controls [44]. PPARγ activation is proposed to retard the initiation and development of hypertension [45] and PAH [46] by mechanisms centered on increased NO bioavailability including 1) inhibition of pro-inflammatory NO-consuming signaling reactions, 2) decreased SMC and EC migration and proliferation, and 3) decreased production of reactive species. The therapeutic potential of PPARγ agonists also reveals promise in animal models of PAH and other vascular disorders. For example, Rosi attenuates chronic hypoxia (CH)-induced right ventricular systolic pressure increases (RVSP), right ventricular hypertrophy, vascular remodeling, and Nox4 expression in mice exposed to 10% oxygen for 3–5 weeks. CH-induced Nox4 expression increased superoxide and H_2O_2 in lung tissue and was blunted by Rosi treatment [47••]. These findings suggest that PPARγ and/or activation by Rosi mediates vascular protective effects by improving endothelial function in part through ROS- and anti-inflammatory-dependent pathways.

NO_2-FA suppress pro-inflammatory reactions

Reactive oxygen species induce gene and protein expression by activating transcription factors such as NFkB [48], which in turn play a role in vascular inflammation associated with vascular disorders. Electrophilic NO_2-FAs adducted the NFkB p65 subunit, resulting in inhibition of DNA binding and repression of NFkB-dependent target gene expression [49]. Consequently, NO_2-FAs attenuate LPS-induced macrophage expression and secretion of pro-inflammatory cytokines, inhibited TNFα-stimulated vascular cell adhesion molecule 1 expression and blocked TNFα-induced adhesion of monocytes to endothelium. Moreover, administration of NO_2-FA during or immediately following an ischemic episode induced profound myocardial protection following coronary artery ligation and reperfusion. This protective effect was mediated in part through the inhibition of the p65 subunit of NFkB and the limitation of downstream pro-inflammatory signaling [17••].

Conclusions

NO_2-FAs are byproducts of NO and NO_2^--dependent oxidative reactions. When exogenously administered at concentrations giving plasma levels 5–10 times greater than found endogenously, these species reduce oxidant stress, inflammation and maladaptive vascular remodeling in a variety of pre-clinical inflammatory injury models. *In vitro*, NO_2-FAs inhibit neutrophil and platelet function, VSMC proliferation, endothelial adhesion molecule expression. LPS-induced macrophage activation and macrophage transmigration [50]. In rodent models the administration of NO_2-FAs either by intraperitoneal injection or subcutaneously-implanted osmotic mini-pumps resulted in inhibition of neointimal proliferation following wire-injured vessels [51] and I/R injury to heart and kidney [17••]. Further evaluation of electrophilic nitro- and keto-FAs may reveal a new strategy for limiting the inflammatory reactions and impaired vascular function characteristic of vascular disorders. A broad range of signaling events would be responsible for these effects, since electrophilic lipids act through a multitude of signaling pathways (Figure 2), including increasing eNOS and Nrf2-dependent gene expression, attenuating VSMC proliferation, inhibiting NFkB-induced inflammatory effects and activating PPARγ-dependent gene expression.

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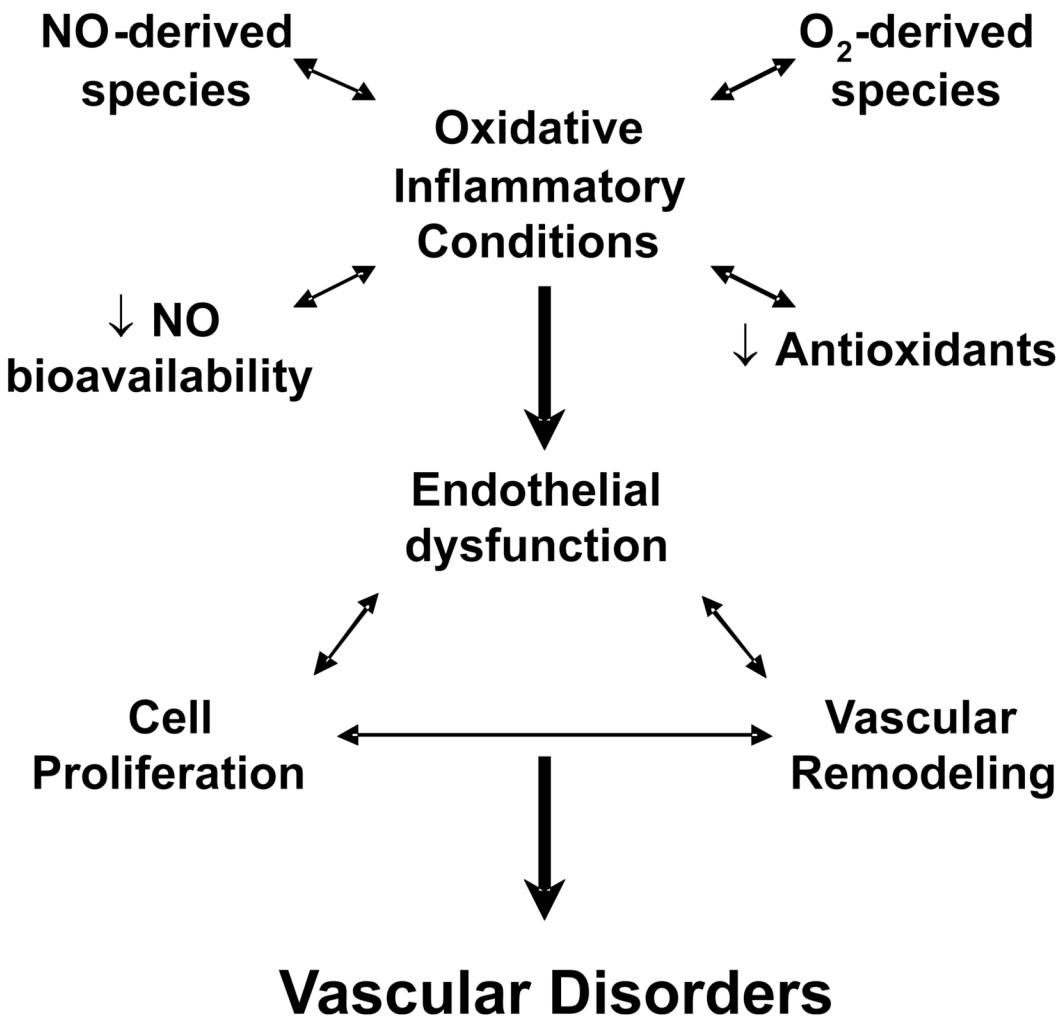

Figure 1.
Mechanisms for oxidative inflammatory-induced endothelial dysfunction, proliferative effects, and vascular remodeling (vicious cycle) in vascular disorders.

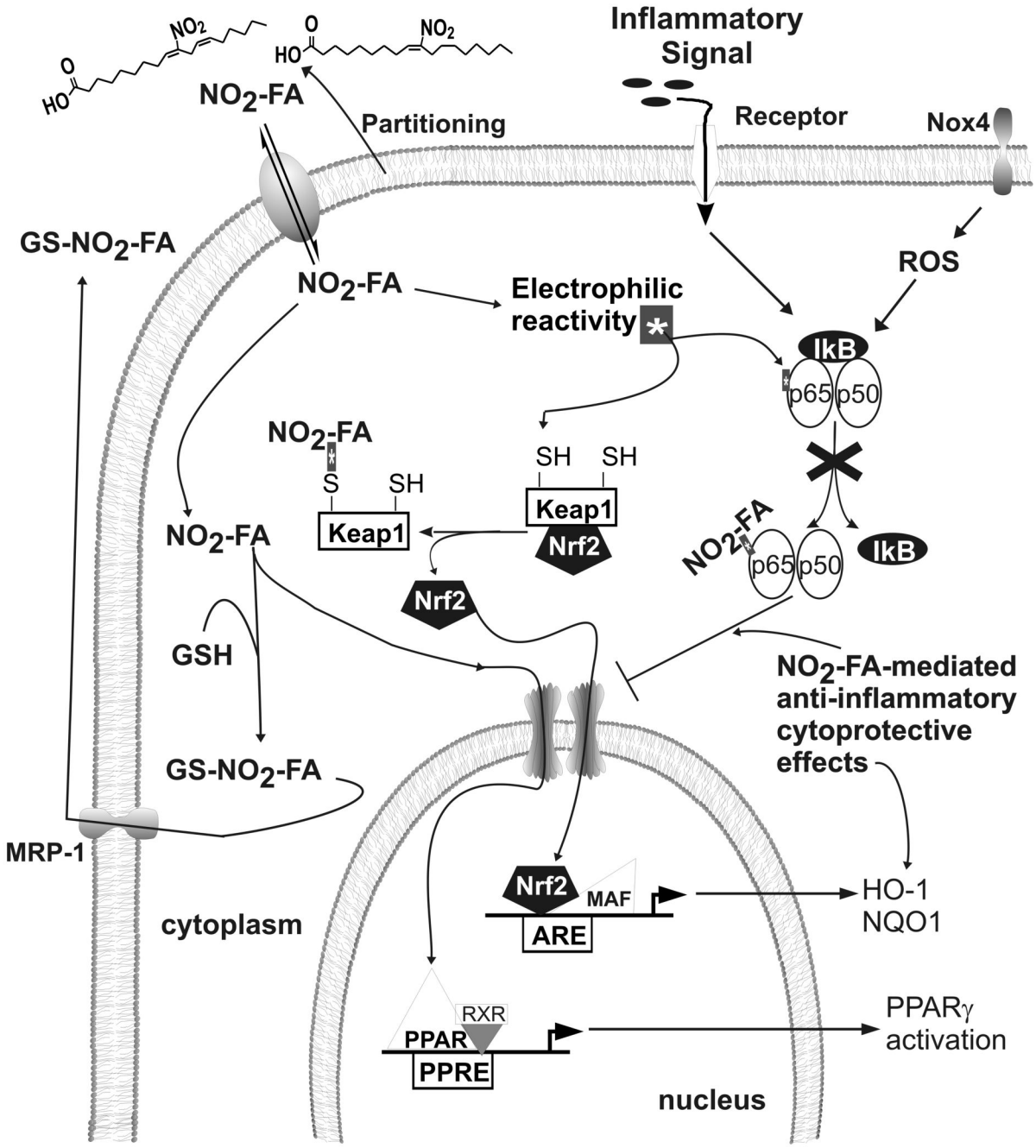

Figure 2.

NO_2-FA-mediated anti-inflammatory and cytoprotective effects. Electrophilic NO_2-FA bind critical nucleophilic residues on p65 and Keap1 thus resulting in inhibition of NFkB-dependent downstream inflammatory signaling and Nrf2-dependent activation of cytoprotective effects, respectively. The disruption of the Keap1/Nrf2 complex by electrophiles leads to Nrf2 translocation to the nucleus forming heterodimeric complexes with small Mafs on the ARE. NO_2-FAs bind to PPARγ and partially transactive PPARγ-dependent gene activation. Electrophile-GSH adducts form in the cytoplasm and can be transported out through specific multi-drug resistance proteins (MRP-1).