[Frontiers in Bioscience, Landmark, 21, 873-889, January 1, 2016]

Nitro-fatty acids in cardiovascular regulation and diseases: characteristics and molecular mechanisms

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1. ABSTRACT

Electrophilic nitro-fatty acids (NO_2-FAs) are endogenously formed by redox reactions of nitric oxide (•NO)- and nitrite ($•NO_2$)- derived nitrogen dioxide with unsaturated fatty acids. Nitration preferentially occurs on polyunsaturated fatty acids with conjugated dienes under physiological or pathophysiological conditions such as during digestion, metabolism and as adaptive inflammatory processes. Nitro-fatty acids are present in free and esterified forms achieving broad biodistribution in humans and experimental models. Structural, functional and biological characterization of NO_2-FAs has revealed clinically relevant protection from inflammatory injury in a number of cardiovascular, renal and metabolic experimental models. NO_2-FAs are engaged in posttranslational modifications (PTMs) of a selective redox sensitive pool of proteins and regulate key adaptive signaling pathways involved in cellular homeostasis and inflammatory response. Here, we review and update the biosynthesis, metabolism and signaling actions of NO_2-FAs, highlighting their diverse protective roles relevant to the cardiovascular system.

2. INTRODUCTION

The discovery of •NO as a free radical mediator of cell signaling and inflammation broadly expanded the scope of redox-regulated cell signaling (1). In addition to activating guanylate cyclase, •NO reacts directly with superoxide ($O_2•^-$) to form peroxynitrite. Homolytic scission of peroxynitrite generates nitrogen dioxide NO_2-FAs and hydroxyl radical, highly reactive molecules involved in nitration and oxidation of biomolecules (2, 3). Nitric oxide thus increases the breadth of reactions that transduce redox signaling. Oxidative inflammatory conditions lead to nitric oxide (•NO) and nitrite (NO_2^-)-dependent unsaturated fatty acid nitration, with both free and esterified fatty acid nitro derivatives being formed (4, 5). Biological fatty acid oxidation and nitration is expected to yield an array of specific regioisomers that can display unique chemical reactivities and signaling actions (6).

Over the last decade, functional characterization and biological functions of NO_2-FAs have revealed clinically relevant protection from inflammatory injury in a number of cardiovascular and metabolic experimental models, including angioplasty-induced restenosis (7), cardiac ischemia-reperfusion (I/R) injury (8), hypertension (9), or atherosclerosis (10). Nitric oxide and NO_2^- biological functions convergence with polyunsaturated fatty acids (PUFA) partially or totally mediate formation of NO_2-FAs species (11, 12).

Pharmacological administration of NO_2-FAs derivatives is entering phase II clinical trials after completing Phase I studies in healthy volunteers and patients with stage 3 and 4 chronic kidney diseases (clinicaltrials.gov #NCT02127190 and #NCT02248051). We will discuss current state of the art and ongoing research efforts associated to the metabolic and cardiovascular benefits of NO_2-FAs and the potential molecular targets for drug development.

3. ADVANCES IN NITRO-FATTY ACID FORMATION AND METABOLISM

3.1. Endogenous generation of nitro-fatty acids

NO_2-FAs display protection from inflammatory injury in various cardiovascular and metabolic experimental models (see below). Formed in the gastrointestinal tract or at the sites of ischemia/reperfusion or inflammation, NO_2-FAs are detected in blood, circulate and distribute to exert adaptive responses in the vasculature and distal tissues and organs. Whereas elevated levels of secondary NOx derivatives attest the amplified oxidation, nitrosation and nitration reactions that occur in inflammation with concomitant increases in tissue damage (e.g. NO_2-tyrosine in atherosclerosis) (13,14), post-translational protein modifications induced by NO_2-FAs are rather salutary (15).

The presence of the endogenously formed NO_2-FAs was originally reported both in normolipidemic and hyperlipidemic donors (16). With levels varying between the picomolar and nanomolar range depending on the mass spectrometry approaches (e.g. LC-MS/MS vs GC-MS/MS), it is undoubtedly evident that selective nitration of unsaturated fatty acids occurs under physiological conditions. Electrophilic nitrated species have been identified and characterized for monounsaturated fatty acids (e.g. oleic acid, NO_2-OA) and polyunsaturated fatty acids (e.g. both conjugated, cLA-NO_2 and bis-allylic, LNO_2 linoleic acid), and arachidonic acid, AA-NO_2) (16-18). However, it has been demonstrated that the reaction of nitrating species with conjugated double bond containing lipids is highly favored when compared to mono (e.g. NO_2-OA) or methyl interrupted dienes and polyenes (e.g. NO_2-LA, AA-NO_2) (19). In this regard, two predominant positional isomers of nitrated conjugated linoleic acid, collectively referred to as cLA-NO_2 occur *in vivo*. These species derive from c9t11-cLA and have nitro groups in either carbon 9 or 12 (referred as 9-NO_2-cLA and 12-NO_2-cLA nitro-conjugated linoleic acid). In addition of mass spectrometric analysis confirming cLA as the major fatty acid substrate for nitration, dietary supplementation with cLA both in animal models and in humans confirms these findings (20). NO_2-FA derived from acidic nitration reactions in the stomach have effects in the vasculature, different organs including liver, kidney, heart, lung and adipose tissue and in immune cells (FJ Schopfer, personal communication). Nitrated fatty acid generated through this pathway results in measurable increases in plasma and tissue levels despite their rapid metabolism (20). Basal levels of NO_2-CLA are in the 1-3 nM range, and are increased to ~10 nM after NO_2^-/CLA supplementation in humans. Despite the controversy with NO_2-OA levels in healthy human volunteers (21), its values are lower than NO_2-CLA and found in the subnanomolar range. NO_2-CLA has been initially found in heart tissue following ischemia reperfusion injury (~10 nM) (22). In addition, NO_2-CLA has been shown to be formed in the peritoneum following LPS injection (23). Yet, increases in systemic circulation stemming from specific organ increases rates of formation have not yet been reported. The rapid metabolism and addition reactions at the site of formation may preclude systemic detection. Of note, recent reports support the notion that electrophilic fatty acids derived from omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), precursors of the inflammatory resolving autacoids (4, 24) are biological substrates for nitration (18).

3.2. Nitro-fatty acid metabolism

The recent discoveries on the convergence of mammalian nitrogen oxide cycle (25) with unsaturated fatty acids provide a strategy to naturally increase endogenous levels of nitro-FA (19). Oral administration of cLA and nitrate yields nanomolar concentrations of nitro-cLA *via* gastric acidification (19). By means of stable isotope labeling of inorganic nitrate (NO_3) and nitrite (NO_2) supplementation in the presence and absence of cLA in healthy human volunteers allowed the determination of NO_2-cLA formation, plasma levels and urinary excretion as well as tissue distribution in animal models (19) (Figure 1). Formation of NO_2-FAs from oral sources of nitrate requires nitrate reductase activity by the oral and gastrointestinal microbiome yielding nitrite, which serves as precursor to reactive nitrating species (26). Nitrite-dependent nitration of cLA is favored by the low pH in the stomach. Nonetheless, the reaction also occurs at neutral pH in aqueous reaction systems, cell models and *in vivo*, catalyzed by peroxidases, globins containing pentacoordinated Fe and nitric oxide autooxidation (23). In experimental pharmacodynamic studies, nitro-cLA reaches a peak in the circulation between 2 to 6 hours post-oral administration following absorption and tissue distribution, including stomach, intestine, colon, plasma, liver, and urine. NO_2-FA are transported back into blood circulation as reversible glutathione (GSH) conjugates (27). Additional metabolism includes hepatic β-oxidation and nitroalkene saturation (28) (Figure 1). Nitro β-oxidation metabolites ((C2H4)n shorter chain nitro-derivatives) are detectable in murine models of inflammation and in healthy human urine (20).

The electrophilic nature of NO_2-FA is subjected to tight metabolic control as revealed by the rapid addition to nucleophilic molecules both in tissues and

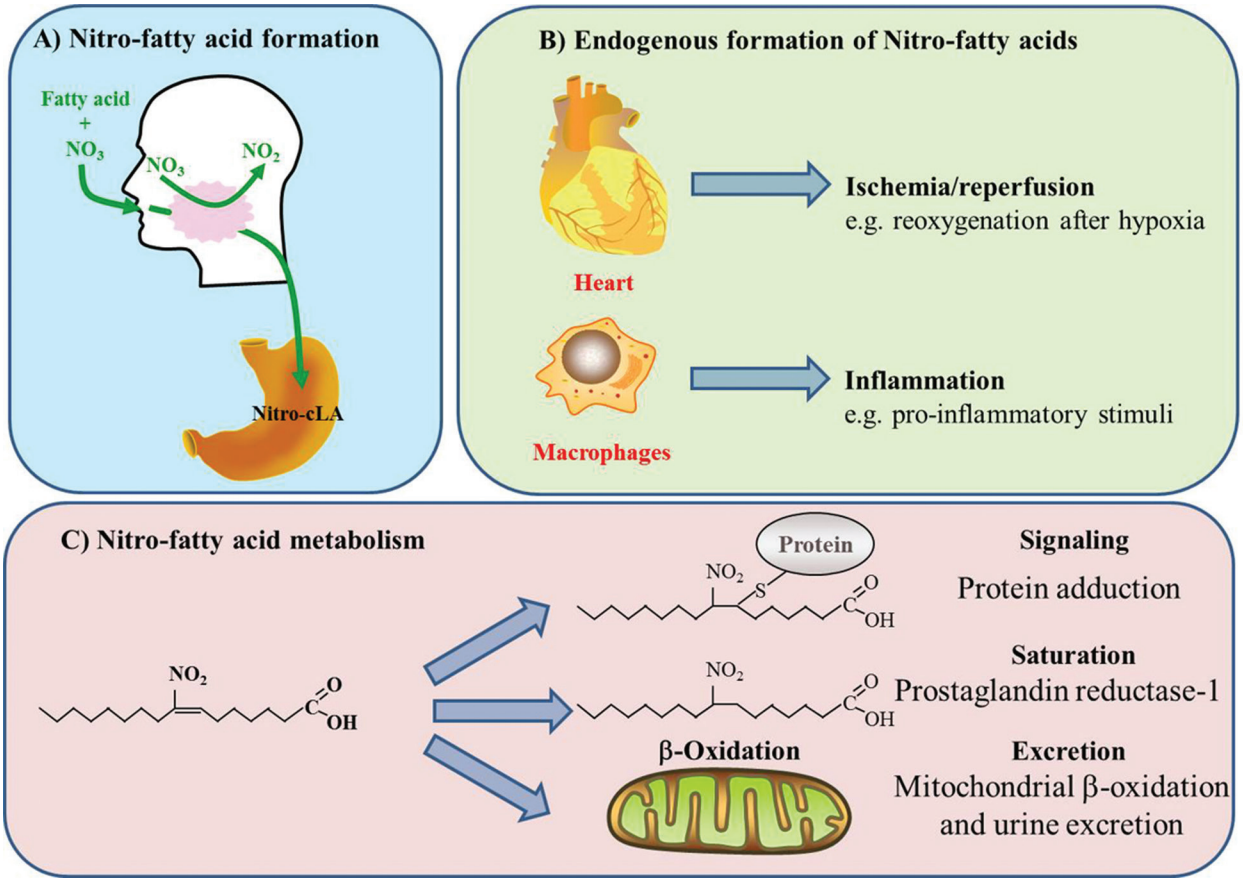

Figure 1. Endogenous NO_2-FA formation and metabolism. (A) Experimental models and human studies have determined that unsaturated fatty acid nitration is readily bioavailable upon oral delivery of PUFA along with inorganic nitrate (NO_3). Oral and gut microbiome mediates NO_3 reduction to nitrite (NO_2) which facilitates formation of NO_2-FAs (19) (B) Nitration derivatives of unsaturated fatty acids are generated in monocytes and macrophages as products of inflammatory-derived reactive species reaction with lipids (11, 12). Oxidative inflammatory conditions lead to nitric oxide (NO) and nitrite (NO_2)-dependent unsaturated fatty acid nitration. Fatty acid nitration products are also generated *in vivo* in the mitochondria of cardiomyocytes following ischemic precondition (8, 22). (C) Post-translational protein modifications induced by NO_2-FAs constitute the primary mechanism of cell signaling *via* Michael addition of cysteine and histidine residues regulating key metabolic and inflammatory processes. NO_2-FAs are the further endogenously metabolized by saturation *via* PtGR-1 and glutathione conjugation and subsequent b-oxidation and ultimately excreted *via* urine (20).

in the circulation, posing analytical challenges in the determination of their non-adducted levels. NO_2-FAs saturation and conjugation to glutathione represent two mechanisms of inactivation, although it has to be noted that the reversibility of glutathione adducts by beta elimination releases electrophilic nitroalkenes. However, reaction constants of NO_2-FA with cysteine residues within proteins, the primary mechanism of signaling action of NO_2-FA (e.g. Michael addition) is orders of magnitude more efficient than that of glutathione (Figure 2). These species are detected upon cLA and nitrate oral supplementation. (20). Recent studies have identified the NADPH-dependent enzyme, prostaglandin reductase-1 (PtGR-1), as centrally involved in the inactivation of NO_2-FAs in the liver (29) (Figure 1). At the cellular level, multidrug resistant protein-1 (MRP-1) serves as a shuttle to regulate intracellular levels of NO_2-FAs and upon glutathione conjugation, NO_2-FAs are actively transported out of the cell (30). However, preclinical and clinical experimental evidence support the therapeutic value of NO_2-FAs yielding adequate endogenous levels to promote anti-inflammatory reactions in the cardiovascular system (31). Furthermore, ongoing studies are expanding our knowledge on the metabolic esterification of free NO_2-FAs into phospholipids, and triglycerides (5).

4. NITRO-FATTY ACIDS IN CARDIOVASCULAR DISEASES

Several reports indicate that nitrated fatty acids can release nitric oxide (32-35). Thus, formation of nitro-fatty acids may serve as reservoirs for nitric oxide in the circulation and on target organs. Nitric oxide release from NO_2-FAs occurs however in aqueous solutions, a process that is inhibited in hydrophobic compartments through nitroalkenes stabilization (e.g. micelles, membranes). In addition, NO_2-FAs induce endothelium-independent vasorelaxation, a mechanism shown to

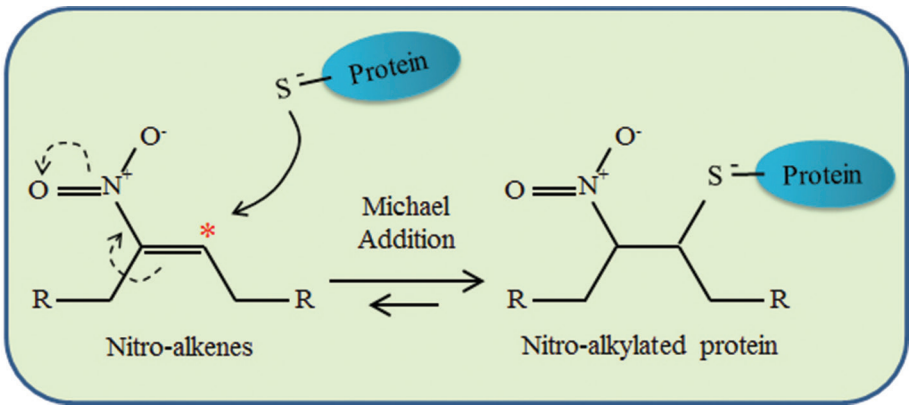

Figure 2. Nitroalkene mediated posttranslational modification (Michael addition reactions). Nitroalkenes react with the thiolate anion of glutathione (GSH) and cysteines (Cys) *via* Michael addition with a reaction constant for OA-NO_2 with glutathione is 183 $M^{-1}s^{-1}$. The reaction with thiols is reversible and in the case of cysteine displays a K_D of 7.5. × 10^{-6} M (20).

involve release of NO (17, 36). However, the primary mechanism and signaling action of NO_2-FA is mediated by posttranscriptional modification (PTM) *via* covalent adduction of NO_2-FAs with functionally-significant thiols *via* Michael addition (Figure 2), with these reactions modulating critical adaptive signaling pathways. These events constitute part of the broader spectrum of the electrophile-responsive proteome, including isoketals that form during inflammation (37, 38). While both group of molecules (nitroalkenes and isoketals) contain an electrophilic group, the aldehyde present in isoketals is a harder electrophile capable or reacting with DNA bases in addition to proteins, in addition of being immunogenic (39). On the other hand, nitroalkene derivatives of linoleic acid (NO_2-LA) and oleic acid (NO_2-OA) were originally identified as agonists of the nuclear lipid receptor peroxisome proliferator-activated receptor-γ (PPARγ) (40, 41), redox-sensitive transcription factors NF-κB (42), Keap1/Nrf2 (43, 44) and heat-shock protein modulation centrally involved in cardiovascular biology (45). The identification of redox-active residues (primarily cysteines and histidines) in key signaling proteins targeted by NO_2-FAs is an evolving field of research. Additional mechanisms have been uncovered in recent years. Figure 3 summarizes current knowledge on PTMs and signaling actions of NO_2-FAs including the activation of cytosolic and nuclear stress-response pathways (44, 45), inhibition of enzymatic activities (e.g. xanthine oxidase) (46), as well as modification of the mitochondrial proteome (47, 48). Thus, these properties of NO_2-FAs revealed their pleiotropic protective actions (Table 1). It will be important to further uncover novel mechanisms regulated by NO_2-FAs and identify whether these novel actions of lipid mediators could translate into protective actions in complex cardiovascular pathologies.

4.1. Nitro-fatty acids in ischemic heart disease

The first demonstration of fatty acid nitration as an adaptive mechanism in the cardiovascular system was revealed in the heart. Using Langendorff-perfused heart subjected to ischemic preconditioning, formation of NO_2-FAs occurred (8). Of note, endogenous generation of nitro-FA was not observed when myocardial ischemia occurs without reperfusion (22). Fatty acid nitration in the mitochondria occurs *via* oxidative mechanisms in a •NO-dependent manner (49). Furthermore, NO_2-FAs limit ischemic injury of the heart. A markedly reduced neutrophil accumulation is observed within the infarct zone in mice treated with NO_2-FAs, concomitant with a reduction of myeloperoxidase (MPO) in the infarct zone (22). NO_2-FAs exert cardioprotection primarily by an anti-inflammatory and anti-oxidant mechanisms *via* adduct formation of critical signaling inhibition of NF- κB (42). Additionally, NO_2-FAs influence the activity of matrix metalloproteinases and may therefore prevent adverse cardiac remodeling directly (50). Ongoing studies are currently pursued to elucidate whether NO_2-FAs play a beneficial role in cardiac function under chronic settings including cardiac hypertrophy models, where signaling events mediated by NO_2-FAs on myocardial growth, matrix remodeling and cell damage, are essential for maladaptive cardiac remodeling (50-52). Of interest, oral treatment with NO_2- and cLA yielding nitro-cLA improves age-dependent cardiac dysfunction (53). This combinatorial approach of cLA and NO_2^- administration also reduces cardiac hypertrophy after myocardial infarction (54). Formation of NO_2-FAs induces the expression of cardiac specific micro RNAs (e.g. miRNA-499) targeting calcineurin and dynamin-related protein-1 centrally involved in mitochondrial dynamics and the apoptotic pathway (55).

In addition, nitroalkylation of a selective pool of proteins in cardiomyocytes may account for the improved mitochondrial respiration in the heart after myocardial infarction (22). For instance, studies by Nadtochiy *et al*. have identified mitochondrial adenine nucleotide translocase 1 (ANT1) as a potential target of NO_2-FA

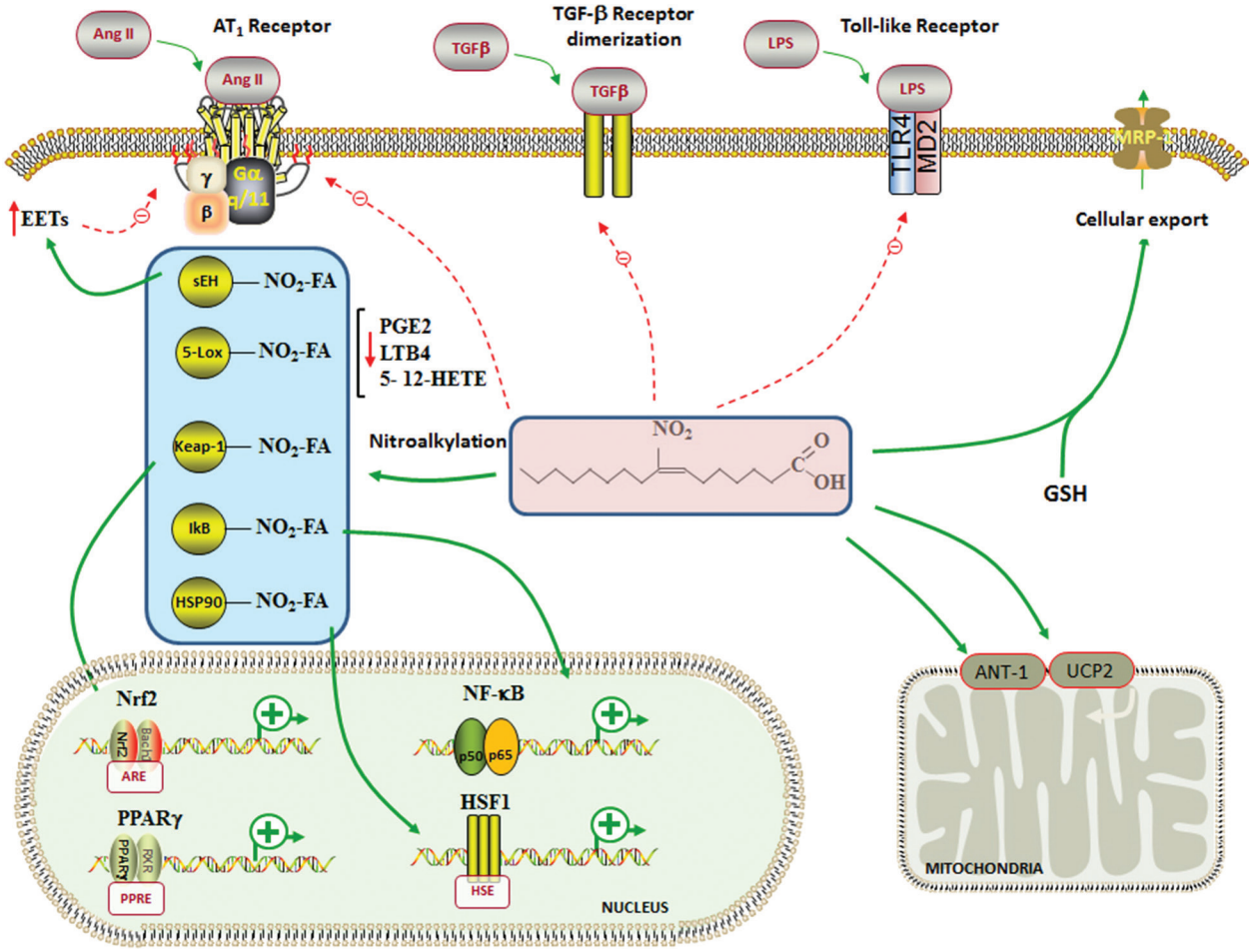

Figure 3. Key posttranslational modifications and signaling pathways regulated by NO_2-FAs. NO_2-FAs mediate posttranslational modification by regulating either their enzymatic activity, membrane receptor interaction with agonists, mitochondrial uncoupling or transcriptional regulation and signaling pathways centrally involved in cellular homeostasis. At the transcriptional level, NO_2-FAs are endogenous PPARγ ligands and regulate PPAR transcriptional activity by selective corepressor displacement and transcriptional activation of peroxisome proliferator activating elements (PPRE) (40). NO_2-FAs regulates the heat shock response by activation of heat shock factors (HSFs), such as HSF1, translocate to the nucleus, trimerize and bind to heat shock elements (HSE) driving the expression of heat shock proteins (45,60). NO_2-FAs bind Keap1 and stabilize Nrf2 transcription factor, which binds to antioxidant response elements (ARE), and regulates antioxidant and detoxifying gene expression (43, 44). NO_2-FAs inhibits the formation of pro-inflammatory lipid mediators (e.g. PGE_2, LTB4, 5- 12-HETE) and promotes anti-inflammatory eicosanoids (EETs) by inhibiting the activity of 5-lipoxygenase (80) and soluble epoxide hydrolase (75), respectively. At the cellular membrane, NO_2-FAs interfere with angiotensin II receptor (9), TGFβ receptor (87) or Toll-like receptor 4 (31) by preventing G-protein coupled receptor second messenger signaling, receptor dimerization or membrane recruitment, respectively.

adduction among the pool of nitroalkylated proteins in cardiac mitochondria (47). A Cys57 modification of ANT1 and post-translational modification of UCP-2 by NO_2-LA is responsible for mild uncoupling of the mitochondria (8). Indeed, mitochondrion-selective derivative of NO_2-FAs have recently devised (56). Mitochondrion-targeted nitro-FA by means of TPP+ modification further increases its cytoprotective efficacy in cardiomyocytes, suggesting that improved mitochondrial bioenergetics maybe the underlying protective role of NO_2-FAs in cardioprotection (57). However, it remains to be established whether this NO_2-FA derivatives possess therapeutic potential in heart disease.

4.2. Nitro-fatty acids in vascular inflammation and atherosclerosis

Electrophilic NO_2-FAs exhibit direct actions on endothelial cells and regulate leukocyte endothelial interactions in isolated cells and *in vivo* (31, 42) (Figure 4). Leukocyte adhesion to cremasteric venules was largely abolished by OA-NO_2, as assessed by intravital microscopy (31). In addition to regulating the vascular production of •NO *via* enhanced expression of eNOS, NO_2-FAs are not only strong inducers of heme oxygenase (HO)-1 expression (58, 59) *via* Nrf2 dependent and independent mechanisms (43-45), but also of heat shock factors regulating a proper unfolding

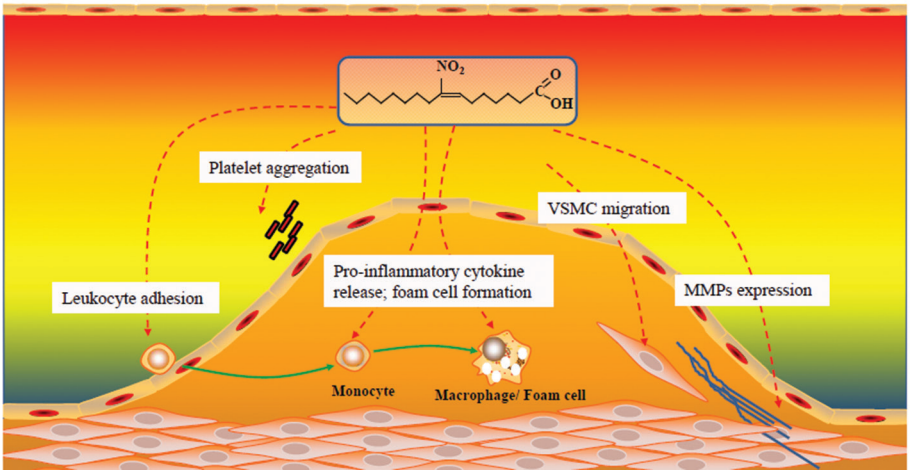

Figure 4. Anti-inflammatory actions of NO_2-FAs in the vasculature. NO_2-FAs act locally in the vasculature by multiple anti-inflammatory processes during the course of atherosclerotic inflammatory responses. These include inhibition of platelet aggregation by limiting the production of pro-thrombotic mediators (71,72), preventing endothelial dysfunction leading to vascular permeability, and reducing leukocyte chemotaxis by inhibiting the formation of pro-inflammatory cytokines (42). NO_2-FAs inhibits monocyte recruitment to the vascular wall during the formation of the atherosclerotic plaque and prevents macrophage activation and lipid accumulation leading to foam cell formation, which further promotes excessive leukocyte recruitment and plaque instability (10). NO_2-FAs reduces smooth muscle cell migration and proliferation (43,58) and inhibits metalloproteinase activity (50) from activated macrophages and migrating smooth muscle cells to the intimal lesion promoting the stability of the plaque's fibrous cap (10) allowing vascular homeostasis to be restored.

Table 1. Nitro-fatty acids in experimental models of disease

Disease model	Actions	Mechanism	References
Myocardial infarction	Reduces infarct size; decreases neutrophil infiltration into the infarct zone; prevents myocyte apoptosis	Inhibition of NF-κB[1] activation; suppression of pro-inflammatory cytokines expression	(8, 22, 49)
Restenosis	Inhibits intima/media thickening after wire injury	Induction of Nrf2[2] signaling and heme oxygenase-1 expression in smooth muscle cells	(7, 43)
Atherosclerosis	Reduces lipid accumulation; promotes plaque stability	Inhibition of adhesion molecule expression; reduced vessel wall infiltration of inflammatory cells and metalloproteinase production; increase α-smooth muscle actin expression	(10)
Hypertension	Reduces pre-established hypertension; delays the onset of angiotensin II induced hypertension	Blockade of the angiotensin II Receptor signaling; increased production of EETs[3]	(9, 75)
Vascular endotoxemia	Reduces leukocyte rolling and adhesion to the vascular wall; inhibits vascular inflammation	Inhibition of TLR4-mediated signaling and NF-κB activation	(31)
Pulmonary hypertension	Reduces right ventricular pressure and vascular fibrosis	Prevention of TGFβ[4] receptor dimerization and signaling	(82, 87)
Chronic obstructive pulmonary disease/allergy	Reduces cigarette smoke-induced inflammation; diminishes glucocorticoid hyper responsiveness	Increases PPARγ[5] expression in airway epithelial cells; inhibition of lipoxygenase activity	(81, 102)
Sepsis-induced pulmonary inflammation	Reduces neutrophil and monocyte mobilization in response to lipopolysaccharide	Inhibition of 5-Lipooxygenase	(80)
Diabetes	Promotes glucose uptake and improves insulin sensitivity in adipose tissue	Activation of PPARγ	(86, 95)
Kidney ischemia/reperfusion	Reduces creatinine levels and plasma urea nitrogen	Reduced renal myeloperoxidase levels	(92)
Inflammatory bowel disease	Reduces colon length, overall body weight loss and bleeding	Activation of PPARγ	(101)

[1]NF-κB: Nuclear receptor-κB, [2]Nrf2: NfE2 related factor 2, [3]EETs: epoxy-eicosatrienoic acids, [4]TGFβ: Transforming growth factor-β, [5]PPARγ: peroxisome proliferator activating receptor-γ

response in endothelial cells by *the novo* synthesis of chaperones (44). Under hypoxic conditions, NO_2-FAs increase expression of hypoxia inducible factor-1a via intracellular •NO production, promoting *ex vivo* endothelial tube formation and angiogenesis (60).

In an experimental model of vascular restenosis, NO_2-FAs reduced vascular hyperplasia after injury (7). Induction of HO-1 expression by OA-NO_2 accounts for the inhibition of vascular smooth muscle cell migration after vascular injury. Induction of Nrf2 transcriptional activation and HO-1 expression may partially account for the inhibition of restenosis after endoluminal injury *in vivo* (61).

Atherosclerosis is a result of an imbalanced lipid metabolism and a maladaptive immune response in the vascular wall. Lipoproteins taken up by circulating and tissue macrophages are cumulatively retained in the arterial plaque, thus further amplifying the inflammatory response by the chronic production of inflammatory cytokines. Failure to resolve the continuous pro-inflammatory stimuli and clearance of foam cells within the atherosclerotic plaque contribute to the vulnerability to rupture (62). In a murine model of atherosclerosis, systemic delivery of NO_2-FAs reduces atherosclerotic plaque formation entailing a reduction of vessel wall inflammatory cells, infiltration and pro-inflammatory cytokine production as well as increasing plaque stability by enhanced a-smooth muscle actin expression in the fibrous cap (10).

Multiple molecular mechanisms support an anti-inflammatory role of NO_2-FAs in atherosclerosis. In addition to the effects of NO_2-FAs on vascular cells outlined above, cellular experiments and *in vivo* evidence indicate that NO_2-FAs display potent actions on isolated macrophages and endothelial cells, regulating production of pro-inflammatory cytokines/chemokines and adhesion receptors. NO_2-FAs inhibit vascular inflammation by interfering with the Toll-like receptor 4 (TLR4) (31), beyond NF-κB inhibition *via* electrophilic adduction of the p65 component (42). Also, NO_2-FAs induce the Nrf2 signaling pathway (43-45), and the JAK/STAT pathway in monocytes/macrophages (63). These signaling events promote an overall anti-inflammatory atheroprotective function, such as regulation of the adaptive immune responses, macrophage polarization and increased phagocytosis, which significantly contribute to the stabilization of the atherosclerotic plaque and resolution of inflammation (64). For instance, activation of PPARγ with TZD (Rosiglitazone) primes monocytes towards an anti-inflammatory phenotype (65), whereas macrophages with a loss-of-PPARγ function display a pro-inflammatory phenotype (66) with detrimental consequences in atherosclerosis. Similarly, deletion of TLR4 favors alternative activated macrophages (67). Also, Nrf2 signaling in macrophages promote a characteristic Mox polarization, with an intermediate phenotype pro-inflammatory and alternative activated macrophages (68), with putative implications for atherosclerosis (69).

Beyond established anti-inflammatory mechanisms mediated by NO_2-FAs on monocyte and macrophages, further consideration both in numbers and the dynamic phenotypes are required to established a therapeutic role of nitroalkenes in atherosclerosis and possibly plaque regression (70). Integrative considerations on the effects of NO_2-FAs on vascular hemodynamics where platelet aggregation play an integral role (71, 72) will need to be considered (73). Inhibition of enzymatic activity of well-established pro-inflammatory mediators such as cyclooxygenase, cytoP-450 monooxygenases in the context of atherosclerosis has not been described. Yet, evidence of inhibition of specific oxygenase activities by NO_2-FAs has been uncovered. In platelets, AA-NO_2 inhibits the oxygenase activity of prostaglandin endoperoxide H synthase I (PGHS-1), but not PGHS-2 (74). Inactivation of PGHSI involves an initial reversible binding event followed by an irreversible inactivation of the enzyme that leads to decreased PGHS-1-dependent thromboxane B(2) formation and a decrease in platelet aggregation.

4.3. Nitro-fatty acids in hypertension

Systemic delivery of NO_2-OA reduces blood pressure in an angiotensin II infusion model of hypertension. At the molecular level, NO_2-OA interferes with angiotensin II signaling by preventing G-protein coupled receptor signaling, thus limiting calcium mobilization in vascular smooth muscle cells. Direct adduction of the angiotensin II type 1 receptor (AT_1R) by NO_2-OA is the underlying cause of the observed blood pressure reduction (9). Subsequent studies have also identified inhibition of soluble epoxide hydrolase (sEH) by electrophilic NO_2-FAs by their adduction to Cys521 proximal to its catalytic center (75) (Figure 3). sEH catalyzes the conversion of arachidonic acid-derived epoxides (EETs) into their corresponding diols. EETs are electrophilic lipids which evolved as intermediate derivatives of arachidonic acid metabolism which plays an important role in blood pressure regulation and overall blood pressure homeostasis with implication for cardiovascular and renal disease (76). Indeed, sEH inhibitors are also rapidly advancing toward clinical use for chronic kidney disease and hypertension. These non-exclusive mechanisms highlight the putative use of derivatives of NO_2-FAs in hypertension therapeutics either as alternatives or in combination with existing angiotensin II receptor blockers (ARBs). This notion may be particularly relevant in diabetic and kidney disease patients in which there is no evident survival benefit from current therapies (77). In this regard, preclinical studies indicate that NO_2-FAs administration in combination with ARBs is renoprotective in experimental models of diabetic nephropathy (78).

4.4. Nitro-fatty acids in pulmonary hypertension and fibrosis

Pulmonary arterial hypertension is a highly prevalent cardiopulmonary disease resulting in progressive remodeling of the pulmonary vasculature. An emergent body of evidence suggest that an altered immune response underlie the development of pulmonary arterial hypertension and endothelial dysfunction along with the growth of the smooth muscle medial and adventitial layers (79).

To test the role of NO_2-FAs in experimental models of inflammatory responses of the lungs, the effects of nitro-FA on LPS-induced lipoxygenase activation were studied in the mouse lung (80). Systemic delivery of NO_2-FAs reduced the development of lung injury concomitant with a reduction of circulating and pulmonary levels of the 5-lipoxygenase (5-LO) products, LTB4 and 5-HETE and 12-HETE. Selective adduction of Cys418 in 5-LO was identified. Specificity of the reaction was further confirmed using 5-LO knockout mice in which administration of NO_2-FAs had no effect on LPS-induced neutrophil or monocyte mobilization in the lungs (80). Similarly, NO_2-FAs administration promotes neutrophil apoptosis and subsequent phagocytosis by alveolar macrophages limiting allergic hyperesponsiveness of the lung (81).

In an experimental model of hypoxia-induced PAH, treatment with NO_2-FAs not only reduces the onset of PAH but also reverse its development under chronic hypoxic conditions (82). NO_2-FAs reduce right ventricular pressure and mediate adaptive responses to increased afterload (e.g. fibrosis and ventricular remodeling) caused by hypoxia thus improving right ventricular function. Further studies show that NO_2-FAs may play an additional role in PAH by promoting lung endothelial cell survival (83) *via* the formation of a transcriptional complex between PPARγ and β-catenin and inducing pro-survival molecules (e.g. apelin) in pulmonary endothelial cells (83). Cohort participants with metabolic syndrome, albeit asymptomatic for cardiovascular disease, present subclinical pulmonary hypertension and right ventricular diastolic dysfunction (84), also observed in experimental models (85). In obesity-induced insulin resistance model, treatment with NO_2-FAs improves right ventricular function (86). It reamins to be established whether improved glucose tolerance by OA-NO_2 maybe the underlying cause of improved pulmonary hypertension yet NO_2-FA treatment was associated with reduced pulmonary xantine oxidase activity, oxidative stress, and pro-inflammatory cytokine levels in the lung.

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal disease without effective therapeutic strategy, largely driven by transforming growth factor β (TGFβ). NO_2-FAs converted TGFβ to inactive monomers in cell-free solution, suggesting an additional mechanism mediated through NO_2-FAs for TGFβ inhibition. *In vivo*, NO_2-FAs alleviated symptom of pulmonary fibrosis in mice (87).

4.5. Nitro-fatty acids in kidney diseases

Renal inflammation and kidney failure are intrinsically associated with cardiovascular diseases and are often the outcomes of chronic inflammatory conditions such as diabetes and hypertension. Similarly, cardiopulmonary and systemic hemodynamics, neurohormonal activation and tissue fibrosis further lead to increased risk for chronic kidney diseases (88). Increasing evidence from acute kidney injury models as well as chronic kidney disease indicates that NO_2-FAs may play an important role in the inflammatory processes associated with renal diseases and failure (89). In a murine adriamycin-induced model of focal glomerular sclerosis, preemptive treatment with NO_2-OA reduces creatinine levels, glomerulosclerosis, tubulointerstitial fibrosis, urinary lipid peroxidation products and renal inflammation (78). Similar outcomes are observed in a cisplatin-induced nephrotoxicity model (90). Inflammatory markers in the kidney (e.g. TNFα, MCP-1, ICAM-1, VCAM-1, and PGE_2) are also attenuated following NO_2-OA treatment in a multiorgan endotoxemia model (91). Furthermore, multiple indices of renal protection support the benefits of NO_2-OA treatment after renal ischemia-reperfusion injury in mice (92).

4.6. Nitro-fatty acids in diabetes and metabolic diseases

Several fatty acid derivatives bind PPARγ with different affinities, although the physiological relevance of these interactions is not always evident. Recently, •NO-derived unsaturated fatty acids were found to be partial agonists of PPARs, with preferential affinity for PPARγ, compared with oxidized fatty acid derivatives (93).

PPARγ agonism by NO_2-FAs manifests unique binding kinetics resulting in co-regulator interactions unique to nitroalkenes (94). This suggests that NO_2-FAs induce physiologic responses that differ from thiazolidinediones (e.g. rosiglitazone, pioglitazone). This precept was solidly reinforced by showing that in leptin deficient *ob/ob*$^{-/-}$ mice, a murine model of type 2 diabetes, blood glucose levels were reduced and insulin sensitivity restored by NO_2-OA administration without accelerated weight gain induced by rosiglitazone administration (95).

The role of NO_2-FAs in the genesis of obesity-associated diseases and type 2 diabetes is however less studied. While NO_2-FAs mediate anti-inflammatory mechanisms, its overall regulation of lipid metabolism is only beginning to be understood. For instance, systemic delivery of NO_2-FAs to apoE KO mice reduces the burden of atherosclerosis and macrophage foam cell

formation without an apparent change in overall lipid metabolism (e.g. LDL, HDL, IDL) (10). Other studies support an active role of NO_2-FAs in lipid metabolism. For instance, pro-inflammatory stimuli in macrophages yield cholesteryl-nitrolinoleate (96). This nitroalkene derivative of cholesterol is also present in lipoproteins in human plasma (97). Furthermore, OA-NO_2 delivery to obese Zucker rats reduces plasma triglyceride, normalizes plasma free fatty acids and significantly increased HDL levels (98). In the DOCA-salt hypertensive mouse model, NO_2-FAs delivery reduces lipid accumulation in the liver (99). NO_2-FAs improve glucose-uptake in high fat diet-induced obesity in mice with minimal effect on adipose tissue. However, NO_2-FAs restores adiponectin levels in visceral fat (86). Additional evidence indicates that NO_2-FAs mediate protective effects in lipoprotein and cholesterol metabolism. In Apo CIII-transgenic pigs, NO_2-FAs inhibit lipoprotein-associated phospholipase A_2 (Lp-PLA_2) expression in peripheral blood mononuclear cells (100). It remains to be established how NO_2-FAs regulates obesity-induced altered changes in metabolic signals, lipoprotein and cholesterol metabolism and their translation into an inflammatory response.

5. THERAPEUTIC PERSPECTIVES OF NITRO-FATTY ACID DERIVATIVES

Cumulative experimental evidence supports the therapeutic role of NO_2-FAs in acute and chronic diseases. In particular, those in which an inflammatory component plays an important role in the progression and or resolution of the disease. Herein, we have outlined those relevant to cardiovascular, pulmonary and renal diseases. Further evidences on the anti-inflammatory actions of NO_2-FAs are included in Table 1 and include inflammatory bowel disease (101), chronic obstructive pulmonary disease (102) or the regulation of transient reaction potential (TRP) A1, C and V1 by NO_2-FAs in nociceptive neuronal cells, centrally involved in the regulation of symptoms of inflammation and pain control by the central nervous system (103-105).

Beyond experimental models, perspectives on the biological functions of NO_2-FAs are constantly evolving. Considerations on the endogenous concentrations achievable on biologicals systems, experimental animals and humans continue to pose important questions on the relevance of the signaling pathways mediated through NO_2-FAs in cardiovascular benefits. Studies on cell specific deletions of key signaling pathways described herein, in particular as they pertain to inflammation, are currently pursued (106). In ongoing studies, it will be important to elucidate how NO_2-FAs regulate metabolic and inflammatory aspects of chronic cardiovascular disease in experimental systems and in humans and evaluate the impact of blocking these pathways for NO_2-FAs therapeutic efficacy.

A diverse class of lipid-based therapeutics is rapidly advancing into the clinical arena, creating novel opportunities for therapeutic intervention in chronic disease including cardiovascular disease (107, 108). As phase II clinical trials on NO_2-FAs are being pursued, pharmacokinetics, pharmacodynamics and effect on biomarkers will determine the most adequate dosing regimen for NO_2-FA-based therapeutics. Additional opportunities are being pursued for the development of NO_2-FAs therapeutics in the areas of slow release and selective delivery to end-target organs.

6. ACKNOWLEDGEMENT

The work in our laboratories is supported by National Institutes of Health grants R01 HL123333 (L.V.), R01 AT006822 (F.J.S.), R01 HL064937 (B.A.F.), R01 HL068878 and R01 HL105114 (Y.E.C.).

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Abbreviations: NO: nitric oxide, NO_3: nitrate, NO_2: nitrite, $O_2^{\bullet -}$: superoxide, H_2O_2: hydrogen peroxide, MPO: myeloperoxidase, PPARg: peroxisome proliferator activating receptor-g, AT_1R: angiotensin type 1 receptor, ARBs: angiotensin receptor blockers, XO: xanthine oxidase, PUFA: polyunsaturated fatty acids, OA: oleic acid, NO_2-OA: nitro-oleic acid, LA: linoleic acid, NO_2-LA: nitro-linoleic acid, cLA: conjugated linoleic acid, NO_2-cLA: nitro-conjugated linoleic acid, NF-κB: nuclear receptor-κB, Nrf2: NfE2 related factor 2, VSMC: vascular smooth muscle cells, EC: endothelial cells, HO-1: heme oxygenase-1, 5-LO: 5-lipoxygenase, LPS: lipopolysaccharide, apo: apolipoprotein, sEH: soluble epoxide hydrolase, EETs: epoxy-eicosatrienoic acids, GSH: glutathione, BMP: bone morphometric protein, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, TLR4: Toll-like receptor 4, ANT1: adenine nucleotide translocase 1, Cys: cysteine, TGFβ: transforming growth factor β, PAH: pulmonary arterial hypertension, PLA_2: phospholipase A2, PtGR-1: prostaglandin reductase-1, PGHS: prostaglandin endoperoxide H synthase, MRP-1: multidrug resistant protein-1, LC-MS: liquid chromatography-mass spectrometry

Key Words: Nitro-fatty acids, Cardiovascular Diseases, Nitrite, Nitrate, Therapeutics, Inflammation, Metabolism, Review

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