Review

Innate Immunity

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Effects of fatty acid nitroalkanes on signal transduction pathways and airway macrophage activation

Melissa L Wilkinson[1] and Andrew J Gow[1]

Abstract

Fatty acid nitroalkenes are reversibly-reactive electrophiles that are endogenously detectable at nM concentrations and display anti-inflammatory, pro-survival actions. These actions are elicited through the alteration of signal transduction proteins via a Michael addition on nucleophilic cysteine thiols. Nitrated fatty acids (NO_2-FAs), like 9- or 10-nitro-octadec-9-enolic acid, will act on signal transduction proteins directly or on key regulatory proteins to cause an up-regulation or down-regulation of the protein's expression, yielding an anti-inflammatory response. These responses have been characterized in many organ systems, such as the cardiovascular system, with the pulmonary system less well defined. Macrophages are one of the most abundant immune cells in the lung and are essential in maintaining lung homeostasis. Despite this, macrophages can play a role in both acute and chronic lung injury due to up-regulation of anti-inflammatory signal transduction pathways and down-regulation of pro-inflammatory pathways. Through their propensity to alter signal transduction pathways, NO_2-FAs may be able to reduce macrophage activation during pulmonary injury. This review will focus on the implications of NO_2-FAs on macrophage activation in the lung and the signal transduction pathways that may be altered, leading to reduced pulmonary injury.

Keywords

Inflammation, macrophage activation, nitrated fatty acid, nitroalkene, signal transduction

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Introduction

Nitrated fatty acids (NO_2-FAs) are endogenously-formed compounds that play a major role in regulating cellular processes, especially those involved in the inflammatory response.[1–4] They are formed via non-enzymatic reactions between unsaturated fatty acids and nitrogen dioxide (NO_2) equivalents.[5–7] Cell and tissue levels of NO_2-FAs can be modulated by diet and oxidative stress.[7,8] Similar to other electrophilic fatty acids, NO_2-FAs act as potent signaling molecules. Under inflammatory conditions, they are formed at higher concentrations in the body as their formation requires oxidative stress.[2,4] Fatty acids with conjugated double bonds are the main targets of this reaction, which has been affirmed via mass spectrometry analysis of human urine.[9] NO_2-FAs are electrophilic in nature, which allows them to interact readily with nucleophiles, such as cysteine thiols, on susceptible proteins.[10–12] Reaction with cysteine residues results in a covalent post-translational modification via Michael addition.[13] Through these modifications, NO_2-FAs are able to invoke pro-survival, anti-inflammatory responses in many different tissues and organ systems throughout the body.[14–17]

NO_2-FAs act on many different signal transduction pathways in which cysteine-containing proteins are involved (Figure 1).

They have been observed altering pathways involved in the initiation of inflammatory responses or migration of inflammatory cells.[4,10,18–20] NO_2-FAs are

[1]Department of Pharmacology and Toxicology, The State University of New Jersey, USA

Corresponding author:
Andrew J. Gow, Department of Pharmacology and Toxicology, Ernesto School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854
Email: gow@pharmacy.rutgers.edu

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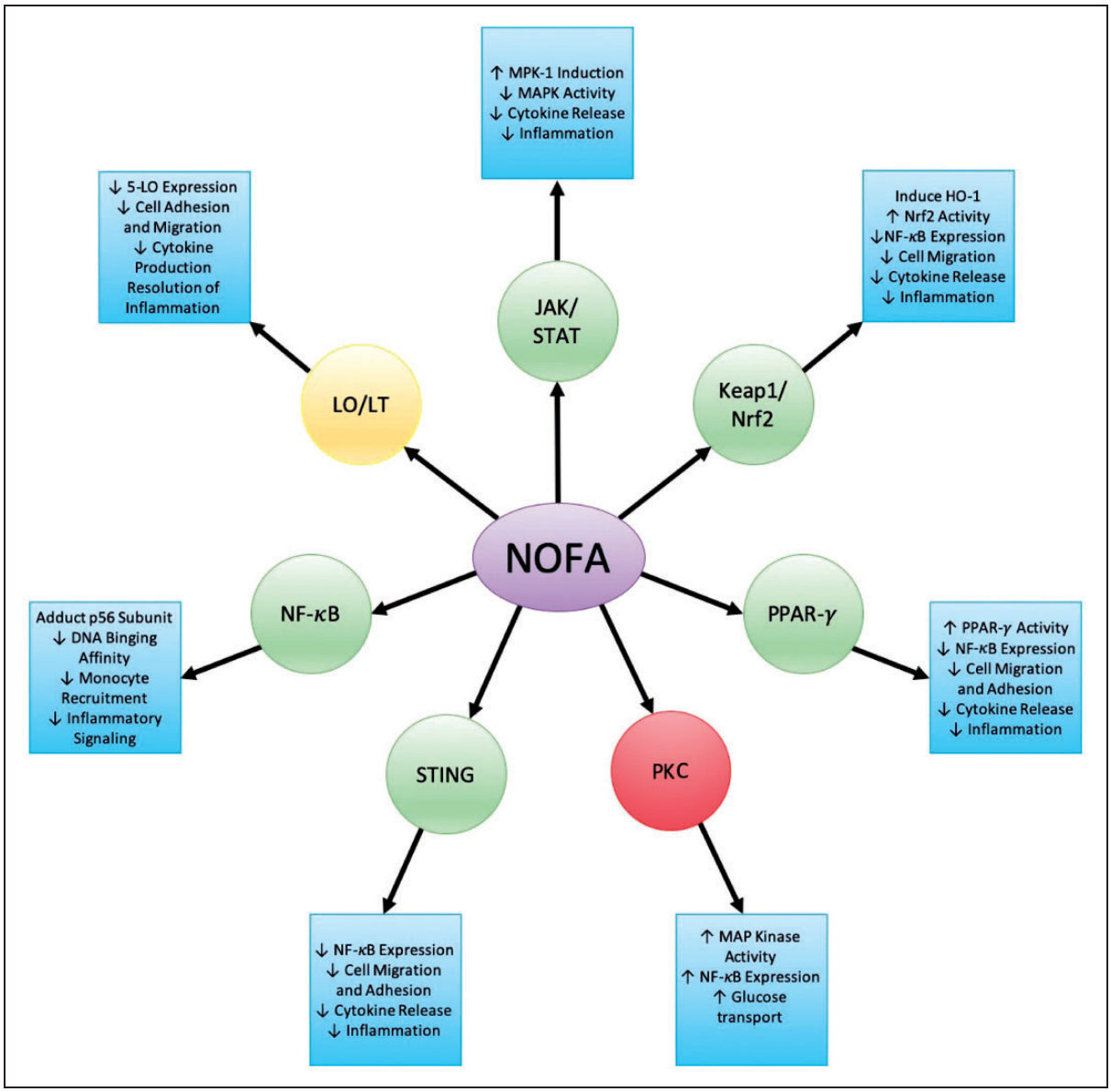

Figure 1. Signal transduction pathways altered by NOFAs and their subsequent anti-inflammatory responses.

pluripotent in nature, meaning that they are capable of altering a number of signal transduction pathways.[21] This makes them ideal anti-inflammatory agents in the lung, as the pulmonary immune system is highly complex and involves many different pathways.

Macrophages are one of the most abundant immune cells in the lung and are essential in maintaining lung homeostasis.[22] Alveolar macrophages are typically resident in the lung and are the first line of defense against pathogens and toxic or allergic particulates.[23] Upon insult, alveolar macrophages will release a wide variety of cytokines and chemokines in response, allowing for recruitment of other immune cells including interstitial macrophages and neutrophils.[24] These macrophages are thought to be classically activated (M1). Airway macrophages also play an important role in the resolution of inflammation and injury.[25] This population is considered to be alternatively activated (M2). Although both populations of macrophages are essential for immune defense and repair in the lung, they can also contribute to injury should their balance be altered.[26] Macrophages play a role in the pathogenesis of both acute lung injury and interstitial lung diseases through an overactivation of the M1 phenotype initially followed by overabundant M2 activation.[26] This will lead to acute inflammatory injury in the lung followed by a progression to fibrosis, should resolution falter.

Macrophage activation and phenotypic switching often occurs due to changes in signal transduction, based on cytokines and inflammatory modulators

present in the lung. Activation may occur via up-regulation of pro-inflammatory pathways, such as the JAK/signal transducer and activator of transcription proteins (STAT) signaling cascade,[27–29] NF-κB,[30–32] lipoxygenase (LO)-dependent leukotriene (LT) synthesis,[33] protein kinase C,[34,35] and stimulator of interferon genes (STING).[36] Macrophage activation can also be inhibited through activation of prominent anti-inflammatory pathways such as the kelch-like ECH-associated protein 1(Keap1)/nuclear factor E2-related factor 2 (Nrf2) pathway[37] and peroxisome proliferator-activated receptor-gamma (PPAR-γ).[38] Alterations to these pathways impact macrophage phenotype greatly and may contribute to their inflammatory and fibrotic potential during lung injury. These pathways are also relevant to NO_2-FAs, in that they all have cysteine residues susceptible to Michael addition.

Through their propensity to alter several signal transduction pathways, NO_2-FAs have shown potential as a therapeutic in a wide variety of inflammatory diseases. They have demonstrated the ability to reduce inflammation in many different organ system pathologies, which may be beneficial in the mitigation or reversal of symptoms.[39] Several studies have been conducted discussing the potential of NO_2-FAs as therapeutics in a variety of organs, including the renal, digestive, nervous, respiratory, cardiovascular, and pancreatic systems.[3,14,40–43] The pulmonary system has not been studied to the same extent, however, they have great potential to mitigate lung injury by balancing macrophage phenotype and activation.

This review will focus on the implications of NO_2-FAs on macrophage activation in the lung. Several of the signal transduction pathways that NO_2-FAs alter will be discussed, along with the mechanism by which these alterations are suspected to occur. These alterations will then be discussed in context of pulmonary macrophage activation and their potential use in reducing lung injury.

Signal transduction pathway alterations and macrophage activation

Several studies have been conducted to determine the mechanism by which NO_2-FAs elicit anti-inflammatory responses. There are three levels at which signal transduction pathways can be modified: the transcriptional level, the extracellular/intracellular signaling level, and the level of signal being transmitted into the cell itself through receptors. The effects of NO_2-FAs on different signal transduction pathways occur through the activation of some pathways and the inhibition of others and many of these pathways are significant in airway macrophage activation. Post-translational modifications to proteins change their signaling abilities and can lead to anti-inflammatory, pro-survival responses through the alterations of macrophage function.

JAK/STAT signaling cascade

The JAK/STAT signaling cascade plays a crucial role in the regulation of bodily defenses against foreign bodies and pathogens via inflammatory response.[4] This cascade is located upstream of the NF-κB, Keap1/Nrf2, and PPAR-γ pathways and is essential in the IL-6 mediated infiltration of T cells during acute inflammation.[44] This pathway is also critical in mediating macrophage responses to cytokines like IL-4, IL-13, and INF-γ and plays an essential role in macrophage phenotypic switching between M1 and M2.[45–47] Ichikawa and colleagues demonstrated that NO_2-FAs are able to inhibit the inflammatory actions of the JAK/STAT signaling pathway in macrophages.[4] NO_2-FAs enhance the induction of mitogen-activated protein kinase phosphatase 1 (MKP-1), which acts as a negative regulator of pro-inflammatory cytokine release.[4] MKP-1 inactivates MAPK such as c-Jun N-terminal kinase (JNK), which are pro-inflammatory in nature.[4] The inactivation of these pro-inflammatory mediators by NO_2-FAs will lead to the inhibition of the STAT portion of the JAK/STAT signaling cascade (Figure 1).[4] Phosphorylation of STATs via JAKs leads to an up-regulation of pro-inflammatory downstream targets such as inducible NO synthase (iNOS) and monocyte chemoattractant protein-1(MCP-1).[27] As NO_2-FAs can inhibit STAT phosphorylation in macrophages, they can inhibit activation of these target genes.[4]

Keap1/Nrf2 pathway

The Keap1/Nrf2 pathway is primarily involved in regulating cellular responses to electrophilic and oxidative stressors.[18,48] Keap1 and Nrf2 both have several cysteine residues that are critical to their function and are susceptible to Michael addition.[49–51] Nrf2 itself plays a crucial role in the activity and coordinated induction of several different antioxidant genes, including phase 2-detoxifying enzymes and other related proteins and antioxidants like GSH, NADPH, dehydrogenase quinone 1 (NQO1), epoxide hydrolase, thioredoxin, catalase, superoxide dismutase, glutamate cysteine ligase, uridine diphosphate glucuronosyltransferase, and heme oxygensae-1 (HO-1).[52,53]

Nrf2 is inactivated when it is bound to Keap1.[54] The nucleophilic cysteine residues on Keap1 provide a suitable target for electrophiles, like NO_2-FAs, and reactions perpetuate conformational changes in Keap1,

allowing for the release of Nrf2 from the complex.[54] Nrf2 release into the cytoplasm allows for increased Nrf2 synthesis.[55] Newly synthesized Nrf2 is able to translocate into the nucleus through the aid of chaperone proteins.[14,55] Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE), allowing for the transcription of genes involved in the regulation of several oxidative stress pathways (Figure 1).[49]

As stated, NO_2-FAs are able to induce Nrf2 dependent, cytoprotective gene expression through its interference with Keap1 binding.[48] NO_2-FAs can covalently adduct Cys237 and Cys288, which are functionally significant in the action of Keap1.[17] Unbound Nrf2 is then stabilized in the cytoplasm and translation is increased,[55] leading to increased production of Nrf2 targets such as superoxide dismutases (SODs), glutathione peroxidase (GPx), glutathione reductase, glutathione-*S*-transferase, NQO1, HO-1, glutamate cystine ligases, and multi-drug resistance protein 1.[48,56–59] Conditions of inflammation or oxidative stress may lead to the activation of certain pro-inflammatory pathways, such as NF-κB, but their activity is disrupted through the activation of Nrf2 by preventing the up-regulation of pro-inflammatory cytokines.[37,60,61] Nrf2 activation by NO_2-FAs will also regulate the migration and infiltration of inflammatory cells through cellular adhesion molecule (CAM) inhibition.[37,60,61]

Nrf2 activation is critical in regulating cellular antioxidants and cytoprotective genes in macrophages.[62] Pro-inflammatory cytokine transcription can be blocked via Nrf2 activation, suppressing inflammatory signaling by macrophages.[37] These anti-inflammatory effects cannot be elicited when Nrf2 is bound by Keap1 as it is marked for degradation. NO_2-FAs have been shown to alter Keap1 binding to Nrf2 *in vivo,* suggesting it may be able to induce Nrf2 signaling.[48,63] This was observed in mouse alveolar macrophages that were exposed to LPS and administered NO_2-FAs, leading to increased expression of several genes downstream of Nrf2-activated ARE, such as HO-1, NQO1, and GCLM.[17] Therefore, OA-NO_2 may elicit anti-inflammatory effects through binding Keap1, leading to decreased cellular migration, cytokine release, and general inflammation via Nrf2-mediated suppression.

NF-κB pathway

NF-κB is a nuclear factor that regulates the expression of genes that encode for pro-inflammatory cytokines during inflammatory responses.[19] Under normal conditions, NF-κB is sequestered in the cytoplasm through binding with NF-κB inhibitory proteins, IKBs.[64,65] During inflammatory conditions, NF-κB is activated via phosphorylation of IKB by the IKB kinase (IKK) complex that is composed of IKK-α, IKK-β, and IKK-γ.[58] These phosphorylation events result in the release of NF-κB and the proteasomal degradation of IKB.[66–69] NF-κB is key in macrophage activation, especially toward the M1 phenotype, and will induce the expression of inflammatory genes such as IL-6 and IL-1β.[70,71]

Because NO_2-FAs are electrophilic, they can adduct the p65 (Cys38) and p50 (Cys 62) subunits of NF-κB, inhibiting its binding to DNA.[17,19] This binding inhibition results in down-regulation of NF-κB target genes, leading to limited downstream pro-inflammatory signaling.[19] Monocyte recruitment is also limited by NF-κB inhibition of NO_2-FAs due to a down-regulation of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Figure 1).[19] Inhibition of the p65 subunit results in the suppression of IL-6, TNF-α, and monocyte chemoattractant protein 1 (MCP-1) secretion, all of which are involved in the inflammatory process.[19] NO_2-FAs can also inhibit phosphorylation of IKB and IKK, resulting in further NF-κB suppression.[69]

NO_2-FAs can also inhibit NF-κB by impairing upstream signaling events such as TLR4 recruitment into lipid rafts in response to injury.[69] Lipid raft microdomains coordinate the initial signaling events for TLR4-mediated inflammatory responses in the membrane.[69,72] During inflammatory events, TLR4 and TRAF6 are recruited to flotillin-1-rich fractions of the membrane.[69] Recruitment to lipid rafts allows TLR4 signaling complexes to activate NF-κB signaling, ultimately leading to downstream signaling of pro-inflammatory effects.[69,73] NO_2-FAs are able to inhibit the recruitment of TLR4 and TRAF6 into lipid rafts, preventing their coordination and signaling, thereby inhibiting NF-κB activity downstream.[69] This has also been demonstrated with other oxidized lipid molecules, like Eritoran, which has been shown to be protective against pulmonary inflammation in influenza.[74]

PPAR-γ pathway

PPAR-γ is involved in the regulation of inflammatory responses, cell proliferation, apoptosis, and metabolic function.[40] PPARs have very large ligand-binding domains compared with other nuclear receptors, giving them the capacity to accommodate and bind many different molecules, including large fatty acids.[75,76] Natural ligands of PPAR-γ include unsaturated fatty acids, 15-hydroxy-eicosatetraenoic acid, 9- and 13- hydroxy-octadecadienoic acid, and prostaglandin PGJ_2.[75] This activation by large lipid ligands results in PPAR-γ heterodimerizing with retinoid X receptor (RXR) and binding to peroxisome proliferator response elements (PPRE) in the regulatory regions of target genes, allowing for their transcription.[40,77] This

results in anti-inflammatory effects. PPAR-γ can also directly react with NF-κB through its p50 and p65 subunits, leading to inhibition of pro-inflammatory signals.[78] PPAR-γ activation in macrophages attenuates expression of several pro-inflammatory genes.[79,80]

NO_2-FAs are potent agonists of PPAR-γ and exhibit anti-inflammatory actions through this reaction (Figure 1).[18,20,81,82] NO_2-FAs are capable of binding all three of the PPAR isomers with high affinity but bind with the highest affinity to PPAR-γ.[20] NO_2-FAs modify PPAR-γ at Cys285 via Michael addition, resulting in partial agonism.[43] This modification may serve to protect Cys285 from inflammatory-derived reactive species as Cys285 is highly susceptible to oxidation.[43] PPAR-γ association with NO_2-FAs additionally promotes interactions between PPAR-γ and NF-κB, leading to further anti-inflammatory effects.[5] In classically active macrophages, PPAR-γ inhibits pro-inflammatory responses, including NF-κB activity, but their expression is typically suppressed in environments promoting M1 activation.[83–85] The ability of NO_2-FAs to activate PPAR-γ and promote its interactions with NF-κB will allow for the pro-survival actions of PPAR-γ to persist and prevent inflammatory activation of macrophages.

LO-LT synthesis

Lipoxygenase-leukotriene (LO-LT) synthesis is a pro-inflammatory process involving phospholipase A_2-dependent hydrolysis of arachidonic acid from membrane phospholipids.[10,86] These phospholipids are oxidized by 5-LO to form the lipid mediators, LT-A_4 and 5-hydroperoxy eicosatetraenoic acid (5-H(P)ETE).[86] This leads to the formation of hydroxyeicosatetraenoic acid (HETE), LT-B_4, and LT-C_4.[86] Formation of these eicosanoids promotes regulation of leukocyte recruitment and activation as they are potent modulators of inflammation.[10,87]

5-LO is the only LO with an appropriate nucleophilic amino acid residue, Cys148, sensitive to undergoing a Michael addition.[86] Cys148 is not within the active site of 5-LO, and modification of this residue by NO_2-FAs results in a non-competitive inhibition of function.[10] 5-LO contributes to inflammatory activation in pulmonary macrophages, promoting cellular migration, cytokine production, and a pro-inflammatory phenotype.[88] NO_2-FAs inhibition of 5-LO function has been seen *in vivo* within the lung following induction of sepsis, resulting in a suppression of the inflammatory response.[10] The overall product formation of 5-LO is inhibited in a concentration-dependent manner, indicating a natural accumulation of NO_2-FAs during sepsis.[10] This leads to the conclusion that perhaps NO_2-FAs act as a feedback mechanism, reducing further inflammatory cell migration and recruitment.[10] It is reasonable to suppose that via 5-LO inhibition, NO_2-FAs can reduce eicosanoid formation, which may be of benefit in lung injury or asthma.[10,87]

PKC pathway

PKCs are a large family of proteins that regulate the function of a variety of other proteins through phosphorylation and are one of the major mediators of signal transduction.[35,89] Their activation leads to the up-regulation of signaling pathways involved in cell adhesion, motility, and inflammation gene regulation.[90,91] There are several ways in which PKCs can be activated, depending on the subfamily to which they belong. Conventional PKCs require calcium and diacylglycerol for their activation, novel PKCs require diacylglycerol alone, and atypical PKCs do not require either substrate for their activation.[35] Once activated, PKCs act as effectors for several tyrosine kinases, cytokine receptors, GPCRs, and adhesion receptors, while activating several signaling pathways like NF-κB, JAK/STAT, MEK/ERK, p38, and JNK.[91–94] Processes will vary depending on the subfamily that is present and the cell type they are located in.[35] All subfamilies of PKC have cysteine residues that may be susceptible to Michael additions.[35]

NO_2-FAs have varied effects on different PKC subtypes and their downstream signaling (Figure 1). The addition of NO_2-FAs to pulmonary epithelial cells has been observed to increase the membrane association of the atypical PKCζ, increasing its overall function.[35] This will induce the MAP kinase cascade, resulting in an up-regulation of NF-κB. Macrophage activation can occur via PKC-ζ signaling.[95] Inflammatory stimuli, such as LPS, will allow PKC-ζ to associate with RhoA, ultimately leading to NF-κB activation.[95] Because NO_2-FAs have the ability to activate PKC-ζ, they may elicit pro-inflammatory activation in pulmonary macrophages. Another PKC that has been observed to be modified by NO_2-FAs is PKC-α. PKC-α is located diffusely throughout the cytosol and will migrate to the membrane in response to secondary messengers such as Ca^{2+}.[96] NO_2-FAs have been observed to prevent this translocation of PKC-α to the membrane, thereby preventing their inflammatory signaling.[96] In macrophages, PKC-α stimulation leads to the release of pro-inflammatory cytokine and NO, contributing to an M1 macrophage phenotype.[97] The inhibition of PKC-α translocation and signaling by NO_2-FAs in macrophages could prevent inflammation and reduce lung injury.

STING pathway

STING is a transmembrane adaptor protein that is activated via binding of cGMP.[98,99] It is located in the endoplasmic reticulum (ER) and the mitochondria-associated ER membrane.[36,100] Binding of cGMP leads to the recruitment and phosphorylation of TANK-binding kinase 1 (TBK-1), which plays a role in innate immune system activation via reactions with PRR.[99,101,102] TBK-1 activates interferon regulatory factor 3 (IRF3), which will homodimerize and relocate to the nucleus.[99,102] The complex will initiate the transcription of several pro-inflammatory genes, leading to increased cytokine production.[99,102] This response induces many type 1 IRF genes to be expressed, which are most common in monocytes and fibroblasts.[102] TBK-1 activation will also lead to NF-κB induction.[103] This will further strengthen the inflammatory response.

A study by Hansen and colleagues demonstrates the potential of NO_2-FAs to inhibit STING signaling via adduction to cysteine 88 and 91, as well as the N-terminal histidine (His18).[99] This adduction will lead to inhibition of STING and deregulation of STING palmitoylation.[99,104] This inhibition will prevent the expression of pro-inflammatory cytokines such as IL-6 and type 1 IRF.[99] Inhibition of STING may also lead to downstream inhibition of NF-κB, as TBK-1 will no longer be activated.[103] This results in anti-inflammatory, pro-survival responses (Figure 1).

Hematopoietic cells, like macrophages, have high levels of STING expression and its signaling is up-regulated in infiltrating immune cells.[100,105] STING is highly up-regulated in macrophages under inflammatory conditions and inflammation was shown to be reduced in STING knockout mice.[106] STING activation will lead to downstream activation of inflammatory cytokines, which will further exacerbate inflammation.[106] Because STING is up-regulated during injury in the lung, NO_2-FAs could be utilized to reduce their response via its adduction.[99,107] This will reduce cytokine levels and NF-κB activation via a failure to activate TBK-1.

Acute and chronic inflammatory diseases in the lung

Macrophages play a key role in several acute and chronic inflammatory diseases and infection in the lung (Figure 2).

The lung is highly dependent on innate immune function to defend against inhaled pathogens and particulates.[23] This first line of defense is highly dependent on resident alveolar macrophages, which account for the vast majority of cells in the lung lining.[22] Macrophages will transition between classically and alternatively activated phenotypes when dealing with these insults and the balance between the two is essential in dealing with the insult and preventing injury.[26] When this balance is altered injury prevails and, because of this, the lung is the perfect system to study the therapeutic effects of NO_2-FAs. Macrophages are abundant in the lung and their response to insult or particulates is heavily reliant on signal transduction. NO_2-FAs have the capacity to alter signaling in several of these key pathways, which may lead to reduced activation and decreased lung injury.

Toxicant exposure, viral and bacterial infections, and idiopathic disease can all elicit injury and disease in the lung. There are few effective treatments once the lung is injured and lasting effects may arise or persist after the initial treatment. Few studies have been conducted describing the potential of NO_2-FAs as therapeutics for these disease states but those that have been conducted have shown promising results.

Acute lung injury

During acute lung injury, pulmonary inflammation is mediated by the innate immune system.[108,109] Acute lung injury can be caused by a variety of exposures to inhaled toxicants and infectious agents leading to decreased lung function and substantial morbidities and mortalities in severe cases.[108,110,111] One model of acute lung injury is through the administration of bleomycin intratracheally.[112] The inflammatory response is macrophage dominant, allowing for the examination of macrophage phenotype in response to injury.[112] It has been demonstrated that NO_2-FAs can reduce acute lung injury in this model, potentially by altering alveolar and interstitial macrophage phenotypes.[112] NO_2-FAs preserve resident alveolar macrophage populations, which are typically lost in acute lung injury,[112] and are thought to maintain the non-inflammatory state of the lung.[113] Within the interstitial macrophage population, NO_2-FAs inhibit pro-inflammatory activation, as evidenced by reduced expression of Ly6C and CD206.[112] This data indicates that NO_2-FAs can suppress alveolar and interstitial macrophage activation, which may be due to alterations in inflammatory signaling.[112,114]

Chronic inflammatory disease

Chronic inflammatory diseases, such as asthma, are also influenced by macrophage activation and may be attenuated by NO_2-FA administration. Asthma is characterized by bronchial hyperresponsiveness, airway remodeling, and recruitment of inflammatory cells.[115,116] Due to their role in regulating pro- and anti-inflammatory responses in the lung, alveolar

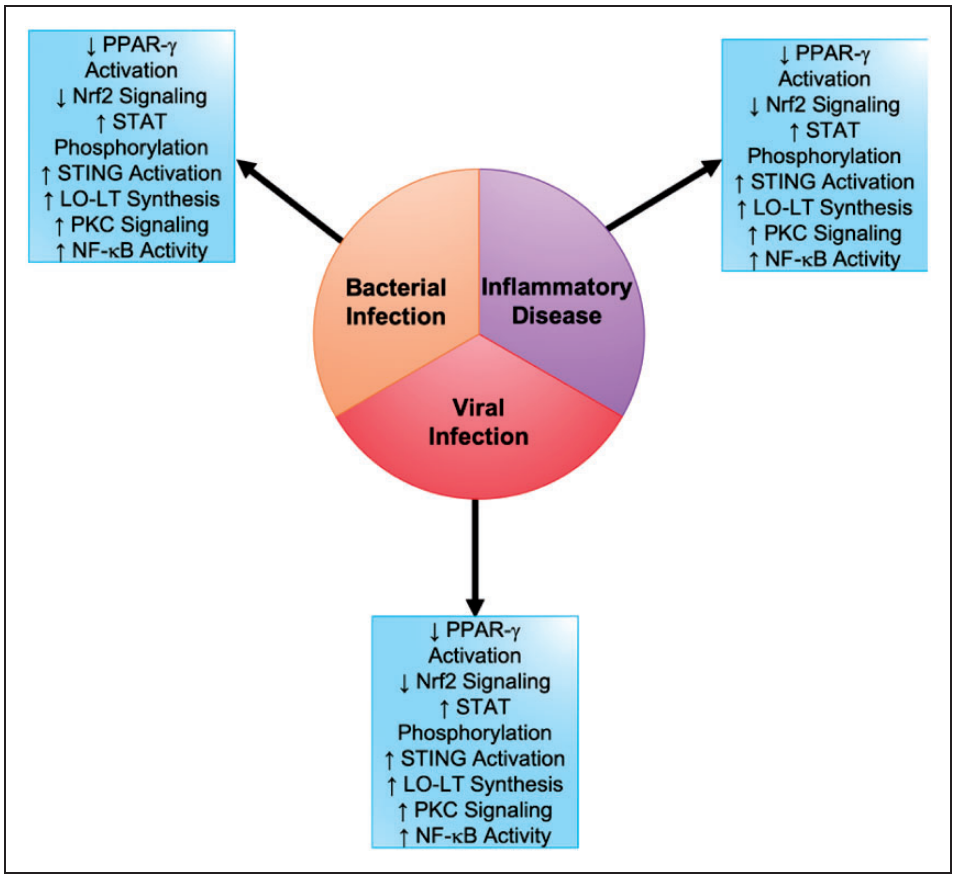

Figure 2. Signal transduction pathway alterations in pulmonary macrophages during inflammatory injury and viral and bacterial infections

macrophages play a critical role in asthma pathology.[116] Because of this, NO_2-FAs may be able to alter this response. It has been observed that NO_2-FAs have the capacity to diminish disease severity in a model of allergic airway disease.[117] Infiltration of inflammatory cells into the lungs is suppressed and phagocytosis of neutrophils by alveolar macrophages is induced.[117] NO_2-FAs also reduce expression of pro-inflammatory cytokines and chemokines, which have the potential to activate macrophages.[117] This data indicates that NO_2-FAs may be beneficial in treating chronic inflammatory conditions via altering macrophage phenotypes.

Viral and bacterial infection

Macrophages also pay a large role in detecting viral and bacterial infections and respond via modulating their protein expression, post-translational modifications, and subcellular locations.[118] Activation of STING in macrophages contributes to inflammation during viral infections.[99] NO_2-FAs have been observed to be up-regulated during viral infections in response to STING activation.[99] INF-1 induction is inhibited with NO_2-FA administration leading to decreased STING activity and reduced inflammation. NO_2-FAs may be relevant in treating certain effects of COVID-19, such as cytokine storm, which is associated with more severe forms of the syndrome.[119] The cytokine storm is characterized by an up-regulation of NF-κB and STAT signaling.[119] These pathways both have the potential to be inhibited by NO_2-FAs, which may lead to reduced disease severity. It should be noted that this is a double-edged sword, as the inflammatory response may be necessary to rid the body of the virus. Because of this, their role as therapeutics in infection should be further investigated.

Discussion

NO_2-FAs are a novel class of compounds that have already shown great potential to modify inflammatory disease states in a variety of organ systems.[5,40,41,120,121] Despite this, their effects on the pulmonary system and macrophage activation is less well known. Based on their mechanism of action, NO_2-FAs may have great potential for reducing injury in pulmonary inflammatory disease states such as acute lung injury and interstitial lung disease. Macrophages are a major

contributor to these disease states and also work through many of the pathways altered by NO_2-FAs. NO_2-FAs have been observed to elicit anti-inflammatory responses in pathways like STING, PPAR-γ, NF-κ-B, Nrf2, and STAT (Figure 1), all of which are prominent in macrophage activation. They are PPAR-γ agonists, leading to the activation and the transcription of a variety of anti-inflammatory genes.[20,82,122] Normally, PPAR-γ is suppressed in classically-activated macrophages but NO_2-FAs may be able to overcome this suppression and allow for more robust activation.[83,84,123] PPAR-γ activation will also decrease cellular adhesion and migration, which may lead to decreased macrophage migration and recruitment during lung injury.[123] This increase in PPAR-γ activation will also lead to downstream inhibition of NF-κB, leading to further inhibition of inflammatory cytokine expression.[5] In addition, this response can be further amplified via NO_2-FA adduction directly to NF-κB[13]. NF-κB is essential in lung macrophage activation, especially toward the M1 phenotype.[70,71] NO_2-FAs readily inhibit NF-κB function, inhibiting the transcription of downstream inflammatory genes and cytokine secretion, resulting in an anti-inflammatory, pro-survival response.[19] NF-κB signaling in lung macrophages may be further truncated via NO_2-FA inhibition of the STING pathway.[99] STING inhibition will prevent TBK-1 induction of NF-κB and will reduce expression of inflammatory type 1 INF genes.[99,103] STING is highly expressed on pulmonary macrophages, especially those that are infiltrating the lung, so inhibition of this pathway may reduce cellular infiltration and expression of inflammatory genes during lung injury.

Nrf2 activation will induce cytoprotective and anti-oxidant signaling in pulmonary macrophages.[62] NO_2-FAs can enhance Nrf2 signaling through binding to Keap1, resulting in higher concentrations of free Nrf2.[37] Nrf2 is then free to translocate to the nucleus, where it transcribes genes to mediate oxidative stress pathways, reducing the inflammatory response.[49] NO_2-FAs are also able to inhibit JAK/STAT signaling, which will promote pulmonary macrophage activation via iNOS and MCP-1 expression.[27] Inhibition of this pathway by NO_2-FAs will result in down-regulation of pro-inflammatory genes and increased production of anti-inflammatory MPK-1.[4] The LO-LT pathway also plays a big role in macrophage migration and cytokine production.[124] NO_2-FAs will bind to 5-LO and prevent eicosanoid formation, reducing these pro-inflammatory responses under injury conditions.[10] Through all of these different mechanisms, NO_2-FAs may be able to reduce excess inflammation in a variety of different pulmonary disease states by altering macrophage signaling.

NO_2-FAs can also induce PKC-ζ, which should be considered with caution when discussing this compound as a therapeutic for lung injury.[35] PKC is a major extracellular signaling molecule in the body and its overexpression could lead to excess inflammation itself through the activation of NF-κB.[35] This may lead to an exacerbated injury response if the signal is magnified to a large extent. Caution is also warranted when considering the effects of NO_2-FAs on other pathways that have not yet been studied. There are many other signaling pathways in the body that contain proteins with cysteine thiols and the administration of NO_2-FAs may cause unfavorable effects in some of these pathways. For example, channel proteins, such as the ryanodine channel, may be negatively impacted by NO_2-FA treatment. The ryanodine channel in the sarcoplasmic reticulum has many calcium channels that contain various cysteine residues that may have the potential to undergo a Michael addition, as they have already shown the potential to be *S*-nitrosylated.[125] These modifications could activate the ryanodine channel, which may lead to unfavorable disruptions in striated muscle function.[125] Although they could be highly beneficial in reducing several inflammatory disease states, including those in the lung, we must proceed with caution to ensure the safety of these new compounds.

NO_2-FAs are exciting and new compounds that have demonstrated potential as therapeutics in many inflammatory disease states, but there is still so much that is unknown. Based on their potential to alter numerous signal transduction pathways, NO_2-FAs could be a highly effective therapeutic in preventing macrophage activation during lung injury. More research is needed to further elicit their effects on macrophage signaling in the lung and to determine if there are any harmful effects that pertain to their use in the lung. That being said, NO_2-FAs have successfully cleared five phase I clinical trials in the cardiac system without any toxicity or interference with Qt intervals, suggesting they do not alter cardiac ion channels.[17] This absence of toxicity furthers the therapeutic promise of NO_2-FAs.[17] With further research, NO_2-FAs could prove to be novel therapeutics in a number of inflammatory lung injuries.

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ORCID iD

Melissa L Wilkinson https://orcid.org/0000-0002-3482-3975

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