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Modulation of Asthma Pathogenesis by Nitric Oxide Pathways and Therapeutic Opportunities

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Abstract

Asthma, a chronic airway inflammatory disease is typically associated with high levels of exhaled nitric oxide (NO). Over the past decades, extensive research has revealed that NO participates in a number of metabolic pathways that contribute to animal models of asthma and human asthma. In asthmatic airway, high levels of NO lead to greater formation of reactive nitrogen species (RNS), which modify proteins adversely affecting functional activities. In contrast, high levels of NO are associated with lower than normal levels of *S*-nitrosothiols, which serve a bronchodilator function in the airway. Detailed mechanistic studies have enabled the development of compounds that target NO metabolic pathways, and provide opportunities for novel asthma therapy. This review discusses the role of NO in asthma with the primary focus on therapeutic opportunities developed in recent years.

Introduction

Asthma, a chronic airway inflammatory disease is characterized by high levels of exhaled NO. Classically, NO binds to soluble Guanylate Cyclase, which generates production of intracellular messenger cGMP, leading to smooth muscle relaxation that results in vasodilation or bronchodilation [1,2]. However, considerable evidence supports that the high levels of airway-derived NO mainly participate in asthma pathogenesis by formation of detrimental reactive nitrogen species (RNS) that mediate inflammation and injury. Increased generation of RNS along with reactive oxygen species (ROS) are well documented in asthma [3,4]. Infiltration of eosinophils and neutrophils in the asthmatic airway introduced increased release peroxidases, e.g. eosinophil-peroxidase (EPO) and myeloperoxidase (MPO) [5]. MPO uses nitrite (an oxidation product of NO) as a substrate to generate the nitrogen dioxide radical [5–7] that can nitrate protein tyrosine and form 3-nitrotyrosine [7–10]. The detection of nitrotyrosine in the asthmatic lung along with identification of modified proteins by mass spectroscopy definitively establishes several biochemical targets of NO [5,7,11]. In contrast, levels of S-nitrosothiols are lower in asthmatic airways as compared to healthy controls [5,12]. Recent studies identify increased expression of S-

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Conflict of interest
The author(s) have no conflict of interest to declare.

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nitroso glutathione (GSNO) reductase is responsible for the loss of S-nitroso-thiols [13]. NO levels may also be affected by arginase enzymes, which metabolize L-Arginine, the substrate for NO synthases. The discovery of the importance of arginine/NO metabolic pathways in asthma offers the opportunity for novel therapeutic strategies to treat asthma.

Enzymes in Asthma Pathophysiology

Nitric Oxide Synthases

In the respiratory tract, resident and inflammatory cells are capable of NO production. NO is generated by oxidation of L-arginine (Fig 1); this reaction is typically catalyzed by the NO synthases (NOS) [14]. NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), of which nNOS and eNOS are constitutively expressed, typically in neuronal and endothelial cells [15]. NO derived from the constitutive NOS along with other NO-adduct molecules, in particular nitrosothiols, are able to release NO at levels that modulate airway as well as pulmonary vascular tone [15,16]. On the other hand NO derived from iNOS, seems to play a primary role as pro-inflammatory mediator. All isoforms are present in the lung. Although eNOS is present in epithelium, iNOS is the predominant enzyme in airway epithelium [17].

The iNOS protein expression levels are increased in asthmatic airway epithelial cells [18,19]. The iNOS expression is mostly regulated at the level of transcription [20–24], and Janus kinase (JAK)/signal transducer, activator of transcription 1 (STAT-1), nuclear factor NF-κβ and AP-1 are essential for iNOS gene expression [15,22,23]. Early studies in mice, with targeted deletions of the three isoforms of NOS, identified a role for iNOS in the disease progression. Ovalbumin (OVA) sensitized and challenged wild-type (WT) mice lung showed significant up regulation of iNOS, whereas iNOS expression was undetectable in similarly treated iNOS knock out (KO) mice. However airway hyperresponsiveness between these two groups was similar. Airway hyperresponsiveness in nNOS-deficient and n/eNOS-deficient mice was significantly less than that observed in WT mice [25]. On the other hand, iNOS KO mice sensitized and challenged with OVA had less inflammatory cell infiltration of the lung, particularly eosinophils, as compared to WT animals [26]. Similarly, pharmacologic inhibition of iNOS-derived NO abrogated bronchoconstriction, inflammation, and remodeling processes in a guinea pig model of allergic asthma [27]. In humans, of the presence of higher than normal exhaled NO is strongly associated with transcriptional activation of the iNOS gene [19,28]. In support of transcriptional control of iNOS and exhaled NO levels, Salam et al showed an interaction of particulate matter exposure, iNOS promoter haplotypes and methylation on exhaled NO levels in children [29]. Recent study also confirms the importance of iNOS in virus-induced asthma which involves Th1 and Th17 responses [30]. These data suggest that iNOS inhibitors may be of utility in asthma. However, although iNOS inhibitors L-NAME [L-NG-Nitroarginine Methyl Ester] and L-NIL [N6-(1-iminoethyl)-L-lysine hydrochloride] cause significant reduction of exhaled NO, neither improve lung function in the asthmatics [31], despite the fact that these inhibitors are protective in animal models. Clearly there are differences between the animal models and human disease, which present challenges to development of potent iNOS inhibitors for the treatment of human asthma.

Arginase

Studies over the past decade indicate that Arginases contribute to the pathophysiology of asthma. Arginases exist in two isoforms: Arginase I and Arginase II. Arginase I is a cytosolic enzyme highly expressed in the liver, where it subserves the urea cycle. Arginase II is localized in the mitochondria; present in most tissues, including airway epithelium, the function of arginase II in cell metabolism is unclear [32–35]. Arginases use L-arginine to

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produce L-ornithine and urea (Fig 1). In cells without a complete urea cycle, arginase II may be responsible for ornthine as a final product, which is used as a precursor of polyamines, glutamate, and proline [32](Fig 1). Arginases may also regulate NO biosynthesis by modulating arginine availability for NOS. Up regulation of Arginase genes expression in lung has been linked to asthma in clinical studies of human patients and in mouse models of OVA-induced airway inflammation [36,37]. Arginase II and I are prominent among the asthma signature genes in the murine asthma model [37]. Similarly, asthmatic patients have higher levels of serum arginase activity and lower levels of serum L-arginine (suggesting greater consumption by arginases) as compared with healthy controls [34]. This led to the concept that Arginase may limit L-arginine availability to iNOS, which may result in superoxide anion production by iNOS, generating peroxynitrite, and consequent protein nitration. Greater synthesis of L-ornithine by arginase may also promote airway remodelling in asthma via greater cell proliferation and collagen production [38]. In support of this concept, arginine bioavailability is correlated with airway obstruction in severe asthma [39]. Breton et al performed a detailed study on DNA methylation of NOS-Arginase pathway and its association with exhaled NO in asthmatic children. Increase in DNA methylation of Arginase II was significantly associated with decrease in exhaled NO in asthmatic children. Differences in exhaled NO were also observed for Arginase I in asthmatics. However, DNA methylation in NOS genes was not associated with exhaled NO [40]. Recently, Yamamoto et al studied the relationship of iNOS and Arginase II expression with asthma severity using bronchial brushing and sputum from healthy and asthmatic subjects. The iNOS protein, exhaled NO and nitrotyrosine (reactive nitrogen species), all were correlated with sputum eosinophils, with the strongest relationships in severe asthma [41].

London's group performed a detailed study with 433 case-parent triads to investigate the association between gene polymorphisms in Arginase I and II and childhood asthma. Arginase I single nucleotide polymorphisms (SNPs) and haplotypes were associated with atopy as determined by positive skin test, and with relative risk of asthma. The association was stronger among children with smoking parents. Altogether the data suggest that genetic polymorphisms in Arginase genes may contribute to origins of asthma and atopy [42].

NO, Nitration and S-nitrosylation

NO and its byproducts nitrite (NO_2^-), nitrate (NO_3^-) and reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^-$) all can execute biologic functions and influence cellular metabolism [43,44]. Hence, all NO products may contribute to asthma pathophysiology. Typically, asthmatics have high levels of exhaled NO and high levels of NO_3^-, and nitrotyrosine in the airway lining fluid. NO reacts rapidly with superoxide ($O_2^{\bullet -}$), generated during asthma exacerbations, to produce $ONOO^-$. $ONOO^-$ may decay to NO_3^- or nitrate tyrosine residues of proteins, leading to structural change and alterations in biological function [5,45]. Hemeperoxidases such as MPO and EPO, use NO_2^- as a substrate to generate 3-nitrotyrosine (Fig 1). Immunohistochemical studies suggest that airway epithelium and eosinophils are major cellular sources for nitrotyrosine in asthmatic airways [5]. Some murine model studies suggest that asthmatic responses can occur independent of iNOS and RNS production [25,46], while others indicate a significant role of iNOS in inflammation [26]. Nitration generally represents a pathophysiological condition, but recent work suggests that enzyme denitrase(s) may reverse nitration events [47]. A role for denitrase enzyme(s) in asthma is currently unknown.

Proteomic survey of nitrated proteins in the OVA mouse model of asthma has revealed several critical proteins in different cellular compartments are modified. Antioxidant proteins (catalase, MnSOD, glutathione S-transferase, antioxidant protein 2) are found to have nitrative modification in asthma [4]. Studies confirm that along with nitration,

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oxidation also plays an important role in the proteins modifications and inactivation [4,48]. NO and $ONOO^-$ are capable of modifying thiol residues of cysteines in proteins to produce S-nitrosothiols (SNO), which can alter protein expression and/or function [49,50]. GSNO and other nitrosothiols are commonly found in airway lining fluid where they contribute to host defense and bronchodilation [51]. A number of studies now link GSNO deficiency to asthma pathophysiology [12,13,52]. RSNO are present at lower than normal levels in the tracheal aspirates of asthmatic children [51,53]. Increased catabolism of GSNO results in the overall lower levels of GSNO in asthmatic airway [51], which is due to greater GSNO-reductase (GSNOR) expression and activity in asthmatic airways [13].

Importantly, NO also attenuates NF-κB DNA binding [54]. Several different NF-kB proteins e.g. IKK, p65 and p50 are regulated by S-nitrosylation, whih is the molecular mechanism by which NO alters NF-κB signaling [49,54,55]. The presence of s-nitrosylated p65 and p50 have inverse relationship with the presence of NF-κB p50-p65 DNA binding and NF-κB-dependent transcription [55]. Thus, the alterations of GSNO/NO metabolism in asthma likely contribute to the pathologic consequences in asthma.

Asthma and Recent Therapeutic Opportunities

NF κb and other signaling molecules to target the arginine/NO pathway

Inhibition of the NF-kB pathway has been an active area of study for the discovery of new agents to treat inflammatory disease [41]. Recently, Licochalcone B and Licochalcone D, along with Licochalcone A, were found to inhibit phosphorylation of NF-κB p65 in LPS mediated signaling pathway. Licochalcone B and Licochalcone D consequently were also reported to reduce LPS mediated NO production [56]. Andrographolide, an active component of the medicinal plant *Andrographis paniculata* may also have therapeutic value in asthma. Andrographolide blocked p65 nuclear translocation and DNA-binding activity in the nuclear extracts from lung tissues of OVA-challenged mice [57], and attenuated OVA-induced eosinophilia and IL-4, IL-5, and IL-13 levels in bronchoalveolar lavage fluid. In parallel, tissue eosinophilia, airway hyper responsiveness and mucus production, and iNOS expression in lung were all reduced by this compound. Fisetin, a bioactive flavonol with a similar mode of action on p65 as andrographolide, also reduces eosinophils, IL-4, IL-5, and IL-13 levels in bronchoalveolar lavage fluid in a murine asthma model [58]. Fisetin also attenuated airway hyper responsiveness to methacoline. These bioactive compounds that reduce iNOS and/or NO and attenuate asthmatic responses all execute their therapeutic value via down regulation of NF-κB pathway, but other pathways that exert control over iNOS may also be effective. For example, epimagnolin and fargesin, which are components of the magnolia flower also inhibit iNOS expression and reduces NO production but via effects on the Extracellular signal-regulated kinases (ERK) pathway [59]. Further work is needed to establish efficacy of signal transduction targeted therapies.

Enzymatic inhibition of NOS

Inhibitors of NOS have been studied for their effect on asthma in animal models as well as humans with varying results. For example, L-NIL effectively caused iNOS inhibition, and reduced exhaled NO in asthmatics within 15 min of oral treatment [60]. The NOS inhibitor 1400W effectively reduced eosinophilia and airway resistance in animal models of asthma, but L-NAME failed to improve airway hyperresponsiveness or airway collagen deposition [27,61]]. Other highly selective iNOS inhibitors (GW274150 and BYK402750) have been shown to be promising in decreasing inflammation in a cigarette-smoke exposure mouse model [31]. This may indicate that specific iNOS inhibition is important to achieve therapeutic utility in airway inflammation. On the other hand, potentiation of downstream NO effects via the phosphodiesterase inhibitor sildenafil effectively relaxes carbachol-

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induced contractions in isolated tracheal rings prepared from a rodent model of allergic asthma, which indicates a cautious approach to NOS inhibitors that may paradoxically also cause loss of bronchodilator effects [62].

Arginase blockade as a strategy for asthma

Morris CR et al has suggested an effective line of treatment might aim at arginase inhibition or oral arginine supplementation to balance arginine utilization in asthma [63]. The pharmacologic inhibitor of arginase, nor-NOHA [L-2-Amino-(4-(2'-hydroxyguanidino)butyric acid], increases NO production as measured by NO_2^- and NO_3^-[64]. Treatment with nor-NOHA attenuates airway hyperreactivity and eosinophilia in the intranasal mite-induced NC/Nga mouse model. Although nitrite and nitrate in the lung remained elevated with nor-NOHA, mRNA for IL-4, IL-5, and IL-13, eotaxin-1 and -2, were reduced. Consistent with this, the numbers of bronchial goblet cells were decreased by nor-NOHA [65]. On the other hand, BEC [(S)-(2- Boronoethyl)-L-cysteine], a highly potent arginase inhibitor, increased protein s-nitrosylation and nitration in a mouse asthma model, suggesting that specificity and potency of inhibitors may be important in altering effects on outcomes in asthma [66].

Augmentation of S-nitrosothiol through blockade of GSNOR

Studies have tested the therapeutic role of S-nitrosothiols in the murine model of allergic inflammation and found that instillation of GSNO suppressed NF-kB activation and bronchial hyperreactivity, but did not significantly alter airway inflammation [67,68]. Since blockade of GSNOR is protective in murine asthma model [13], Foster et al recently explored the possibility of using GSNOR inhibitors, GSNORi, to treat asthma [67]. GSNORi raised levels of S-nitrosylated proteins in cytokine stimulated murine macrophage cells and interestingly lowered levels of other immunomodulators, *e.g.* osteopontin, cyclooxygenase-2, and iNOS [69]. Sun X et al have identified a pyrrole based potent and novel GSNORi, N6022, that lowers GSNOR activity, and leads to bronchodilation in the OVA induced asthmatic mouse model [70].

Summary

Alterations of NO and its end products are well delineated in asthma. A multitude of studies have revealed detrimental effects of RNS and beneficial functions of S-nitrosothiols in asthma. Thus, design of compounds to target the arginine/NO pathway in asthma will optimally preserve, or augment, the production of S-nitrosothiols and reduce the formation of toxic RNS. Further detailed studies of NO metabolism will enable innovative treatments that reverse pathologic metabolism in the airway, and recover beneficial products of NO.

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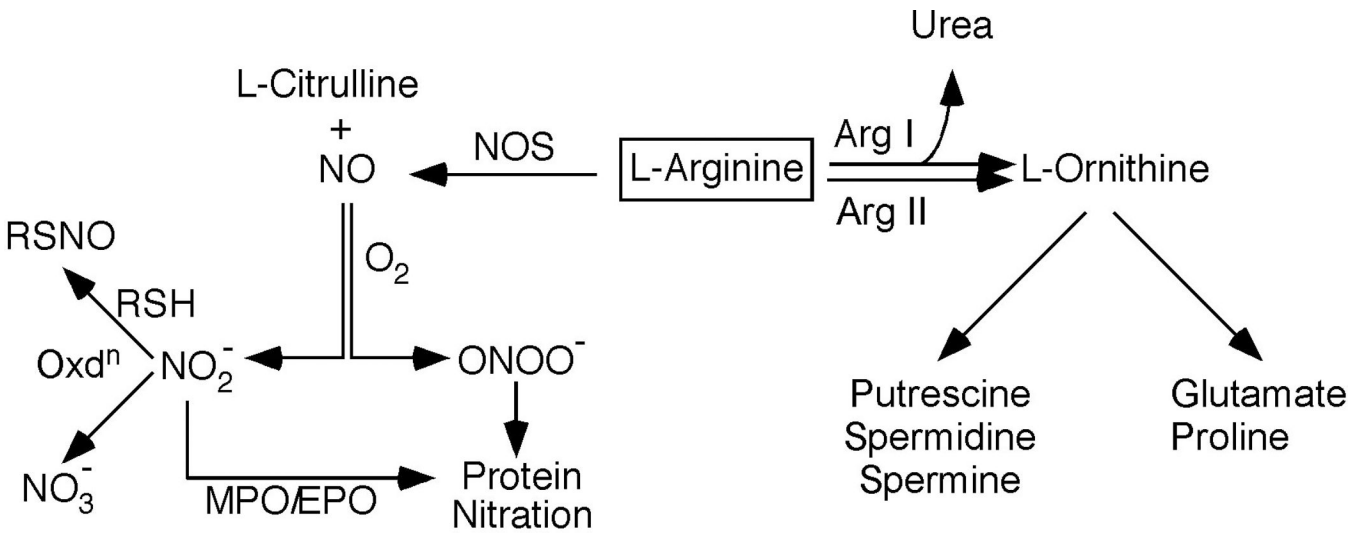

Fig.1.

Metablism of **NO**. Nitric oxide synthases use L-Arginine as substrate to produce NO and L-Citrulline. L-Arginine is also consumed by Arginases I and II to generate urea and L-ornithine. L-ornithine serves as a precursor for Glutamine, Proline, Putrescine, Spermidine and Spermine, which are important to cell proliferation and extracellular matrix formation. NO can be rapidly oxidized to nitrite (NO_2^-), which can be further oxidized to nitrate (NO_3^-). Under disease conditions with superoxide generation, NO can rapidly oxidize to peroxynitrite ($ONOO^-$), which can readily nitrate tyrosine residues of proteins. Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) can also use nitrite as a substrate to nitrate protein tyrosines. In the presence of thiols (RSH), nitrite undergoes nitrosylation reaction to generate S-nitrosothiols (RSNO).

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Table 1

Compounds that Target the Arginine/NO Pathway

Molecular Target	Compounds	Biological Effect	References
NF-kB p65	Licochalcone, Andrographolide, Fisetin	Reduce NO production, attenuate airway hyperresponsiveness, mucus production, eosinophilia	[56–58]
ERK pathway	Epimagnolin, fargesin	Reduce NO production	[59]
NOS	L-NIL, 1400W	Reduce exhaled NO, eosinophilia and airway resistance	[27,60]
Arginase	Nor-NOHA	Attenuate airway hyperreactivity, eosinophilia and mucus hyperplasia	[65]
NO-cGMP-K+channel	Sildenafil	Reduce tracheal hyperresponsiveness	[62]
GSNOR	GSNO instillation, GSNORi, N6022	Increase S-nitrosylated proteins; reduce iNOS levels, attenuate bronchoconstriction	[67,68,70]