**Minireview**

**Inducible Nitric Oxide Synthase (iNOS): Role in Asthma Pathogenesis**

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Asthma is one of the most common chronic inflammatory disorder of the airways of the lungs, affecting more than 300 million people all over the world. Nitric oxide (NO) is endogenously produced in mammalian airways by nitric oxide synthase (NOS) and is known to regulate many aspects of human asthma, including the modulation of airway and vascular smooth muscle tone and the inflammation. Asthmatic patients show an increased expression of inducible nitric oxide synthase (iNOS) in airway epithelial cells and an increased level of NO in exhaled air. Using various NO inhibitors (non-specific or iNOS-specific) and gene knock-out experiments, controversial results have been obtained regarding iNOS’s beneficial and deleterious effects in the disease. In the present review, we have attempted to summarize the results of these experiments and also the genetic studies being undertaken to understand the role of iNOS in asthma. It is argued that extensive biochemical, clinical and genetic studies will be required to assess the precise role of NO in the asthma. This may help in designing selective and more potent iNOS inhibitors and NO donors for developing novel therapeutics for the asthma patients.

**Keywords:** iNOS, Asthma, Nitric Oxide, NOS inhibitor, Gene

**Introduction**

Asthma is a common potentially life-threatening disease of complex inheritance often chronic and disabling\(^1\). Due to rapid industrialization and urbanization, its prevalence is predicted to increase more rapidly in the coming years. Although limited data is available on the asthma prevalence in India, according to the *Global Burden of Asthma Report*, the increase is likely to be dramatic, particularly in India. A wide variation ranging from 4-19% is reported in the prevalence of asthma in school-going children from different parts of India\(^2\). The prevalence of current-wheezing in children in Delhi is 16.7% and the cumulative prevalence is 20.8%\(^3,4\). Another study conducted in Bangalore has reported the prevalence as high as 29.5%\(^5\). The pathological condition results from a complex interaction between genetic and environmental factors.

Clinically, asthma may be categorized into extrinsic and intrinsic asthma. Extrinsic asthma is mainly childhood disorder, though the age of onset can vary and is associated with positive skin prick test (atopy), whereas intrinsic asthma is observed, where the age of onset is above 45 years and is mainly due to age-induced changes in the lung physiology\(^6\). Rather than being a single disease, atopic asthma is currently considered to be a group of different disorders characterized by these major features: (i) elevated immunoglobulin E (IgE) levels, (ii) airway inflammation caused by the infiltration of inflammatory cells including mast cells, eosinophils, basophils, Th2 cells, macrophages and neutrophils which act as source for the release of various mediators including nitric oxide (NO); (iii) bronchial hyper-responsiveness (BHR), which is defined as an increased sensitivity to bronchoconstrictors, such as histamine or cholinergic agonists like methacholine, (iv) intermittent and reversible obstruction of the airways of the lungs, leading to recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night or early in the morning, and (v) subepithelial fibrosis leading to a sequence of changes known as airway remodeling\(^7,8\).

The process underlying the disease pathogenesis is complex and endogenous mechanisms may exist to protect against these processes\(^9\). The airway-derived NO may be important in this process, as it has a potent bronchodilator action by inducing relaxation of airway smooth muscles\(^10\). Also, in a mouse model of
asthma, S-nitrosothiol (SNO), a relatively stable product of NO and thiol is a potent endogenous bronchodilator. Inhibition of endogenous NO has also been shown to increase the airway responsiveness (AHR) and histamine in asthma patients. On the contrary, there are significant evidences to support the detrimental effects of NO. As mentioned above, airway injury by inflammatory mediators including NO has an important part to play in the asthma. In fact NO is being considered as inflammomter in asthma, as its fraction in the exhaled air (FE NO) is increased in proportion to bronchial wall inflammation and eosinophilia or induced-sputum eosinophilia as well as to AHR. FE NO levels are reduced in a dose-dependent manner with anti-inflammatory treatment, thus its measurement has been suggested for the maintenance doses of inhaled corticosteroids. In the present review, we have discussed the dual role of NO in the asthma with major emphasis on iNOS.

NO: A Historical Perspective

Nitric oxide is a small free radical that was previously considered only as an atmospheric pollutant produced by the burning of fossil fuels that contributed to smog and acid rain. However, during 1980s, it was discovered as an agent responsible for the vasodilatation of arterioles and was initially described as “endothelial-derived relaxation factor”. Since then, more than 50,000 publications have appeared on this molecule. Interestingly, it was crowned as ‘molecule of the year’ in 1990. The intellectual jump equating endothelial-derived growth factor with NO resulted in the Nobel Prize being awarded to Furchgott, Ignarro, and Murad in 1998. Since then, NO has been ascribed roles in virtually every body system, with functions as diverse as smooth muscle relaxation, platelet inhibition, central and autonomic neurotransmission, tumor cell lysis, bacterial killing and stimulation of hormonal release. Furthermore, it is involved in the pathogenesis of septic shock and in neuronal damage in stroke and neurodegenerative diseases. Excessive production of NO may contribute to tissue damage in arthritis, glomerulonephritis, diabetes, whooping cough, viral and autoimmune encephalitis and ulcerative colitis. Also, interesting evidences suggest a role for NO in regulation of pulmonary functions and in pathophysiology of pulmonary diseases.

In biological systems, NO and citrulline are produced from the conversion of L-arginine by the enzyme NOS. The reaction requires several cofactors, including flavonoids like flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), heme, calmodulin, and tetrahydrobiopterin (BH4) and the co-substrates are L-arginine, NADPH and oxygen. Arginine derivatives like NG-nitroarginine-L-arginine (L-NMMA), nitro-L-arginine methyl ester (L-NAME), and N omega-nitro-L-arginine (L-NOARG) competitively inhibit this reaction. The NO produced may act directly or could be oxidized variably to nitrite (NO2−), nitrate (NO3−), and peroxynitrite (ONOO−) ions. In fig. 1, NO isoforms are generally classified as either ‘constitutive’ and calcium-dependent or ‘inducible’ and calcium-independent. There are two constitutive NOS including NOS1/neuronal NOS (nNOS) and NOS3/endothelial NOS (eNOS). Inducible NOS includes NOS2A/iNOS (Table 1). The iNOS differs from cNOS in several important ways, namely being independent of elevated calcium, a high-output isoform, having sustained activity for many days, being inducible by a variety of signals, such as pro-inflammatory cytokines, including interferon γ (IFN-γ), interleukin 1β (IL-1β) and tumour necrosis factor alpha (TNF-α) and is inhibited by glucocorticoids.

Suggestive Mechanism of NO Paradox in Asthma

As stated above, a controversy exists about the pro- and anti-inflammatory role of NO in asthma. It has been hypothesized that NO may influence the balance of airway hyperresponsiveness in the following manner (Fig. 2). Receptor stimulation leads to an increase in intracellular Ca2+ concentrations within epithelial cells, which activates the eNOS to generate NO from L-arginine. The NO then binds to soluble guanylyl cyclase at its heme moiety and induces a conformational change, leading to an increase in the activity of the enzyme. This results in an increase in the production of cyclic guanosine 3′, 5′-monophosphate (cGMP) in the target cell, which in turn brings about the relaxation of smooth muscles by a mechanism not yet fully understood. Suggested mechanisms include sequential inhibition of inositol...
Table 1—NOS Nomenclature and function

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Expression</th>
<th>Chromosomal localization</th>
<th>Major function</th>
<th>NO production</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS (cNOS, NOS1)</td>
<td>Neuronal cells, skeletal myocytes</td>
<td>Constitutive</td>
<td>12q24.2</td>
<td>Regulation of neurotransmission</td>
<td>Low</td>
</tr>
<tr>
<td>iNOS (NOS2A, HEP-NOS*)</td>
<td>epithelial cells, smooth muscle cells, macrophages, cardiac myocytes, endothelial cells</td>
<td>Inducible</td>
<td>17q12</td>
<td>Levels are raised in various pathological conditions including asthma and leads to cell death, tissue damage</td>
<td>High</td>
</tr>
<tr>
<td>eNOS (NOS3, ecNOSδ)</td>
<td>Vascular endothelial, alveolar epithelial cells</td>
<td>Constitutive</td>
<td>7q35-36</td>
<td>Vascular tone and structural regulation</td>
<td>Low</td>
</tr>
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HEP-NOS, Hepatocyte NOS; δ endothelial constitutive nitric oxide synthase

Fig. 2—Mechanism of NO paradox in asthma

 triphosphate (IP3), followed by inhibition of cAMP phosphodiesterase, dephosphorylation of myosin light chain, stimulation of membrane Ca$^{2+}$ ATPase, activation of protein kinases, inhibition of calcium influx, and increased sequestration of cytosolic calcium$^{35}$. The bronchoprotective roles of NO independent of cGMP, resulting from S-nitrosylation of ion channels, receptor systems, and other myocyte proteins have also been suggested$^{36}$.

The high concentrations of NO produced by iNOS in epithelial or inflammatory cells after stimulation with cytokines or endotoxin may suppress the activity of cNOS or desensitize guanylyl cyclase. Both phenomenon lead to a diminished cGMP production, which enhances intracellular Ca$^{2+}$ concentrations, thus causing airway contractions (Fig. 2)$^{35}$. NO produced by iNOS also causes dilatation of bronchial vessels and increases plasma exudation. It acts on submucosal glands to cause mucus secretion$^{37}$. It has been proposed that it may act on Th1 cells to down-regulate IFN-γ production, while acting on Th2 cells to upregulate their production of IL4 and IL5$^{38}$. In turn, IL4 increases IgE expression, while IL5 recruits eosinophils into the airways$^{8}$. NO may also mediate cytotoxic effects on airway epithelial cells$^{38,39}$, and may inhibit cell replication by inactivation of enzymes like ribonucleotide reductase$^{34,39}$ or inhibit mitochondrial electron transfer$^{39,40}$. Although there is
a general consensus about the detrimental role of NO produced by the activity of iNOS, we have tried to exemplify it on the basis of various knock-out or genetic studies in experimental animals or humans.

**iNOS Inhibitors and Knock-outs in Experimental Asthma**

To substantiate the role of iNOS in asthma, a number of studies have been undertaken where iNOS function has been eliminated either by specific-inhibitors or by molecular methods by deleting the iNOS gene, but the reports are conflicting. The treatment of the animals with non-specific inhibitors of NOS such as L-NAME, aminoguanidine or NMMA significantly reduces the ovalbumin (OVA)-induced pulmonary eosinophilia. In contrast, treatment with L-N6-(1-iminoethyl)lysine (L-NIL), a selective iNOS inhibitor has shown no effect on airway eosinophilia. In addition, expression of iNOS mRNA or iNOS protein is not increased in the lungs of OVA-challenged mice when compared to control mice. Also, the nitrite levels measured in BAL fluid are not increased. These results suggest that NO contributes to OVA-induced eosinophilia but its production is not generated through the activity of iNOS.

In contrast, in another study, using a similar approach two different selective inhibitors of iNOS namely S-ethylisothiourea (EIT) and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), administered during the challenge period markedly reduced the AHR and BAL eosinophilia. In addition, allergic mice presented an increased NOS activity mainly Ca²⁺-independent, suggesting the involvement of iNOS. Moreover, lung sections of sensitized and OVA-challenged mice were found to be positive for iNOS immunostaining, whereas from PBS-challenged mice, no such staining was observed. Positive iNOS staining is found to be present mainly in the cells among the inflammatory infiltrate. The study has also shown that iNOS promotes airway lung inflammation via direct up-regulation of chemokine expression. The inhibition of NO using non-specific NOS inhibitors amplifies bronchoconstriction and increases collagen deposition in a guinea pig model. However, blockage of only iNOS using 1,400 W, a specific inhibitor attenuates bronchoconstriction, eosinophilic and mononuclear cell recruitment, and collagen and elastic fibers content in airways and thus the remodeling processes.

Strikingly, studies in mice with targeted deletions of the three isoforms of NOS have also shown conflicting results. Xiong et al showed that infiltration of inflammatory eosinophils, loss of structural integrity of the airway walls, microvascular leakage, pulmonary edema, and airway occlusion, but not AHR were markedly less severe in the iNOS mutants than in wild type animals. In contrast, another study found that total NOS activity is increased in sensitized and OVA-challenged wild-type animals as well as in NOS1 and 3 double knock-out (KO) animals, but not in iNOS-KO mice. These results indicate that the enhanced NOS activity detected in the lungs of allergic mice is dependent of iNOS expression. However, no significant differences are observed in AHR and eosinophilic inflammation when comparing wild-type animals with iNOS-deficient mice.

No clear-cut explanation has been provided for the contradictory results obtained relating the regulation of iNOS activity in experimental asthma; however, a role of lipopolysaccharides (LPS) contamination of allergens has been suggested as key element to it. Upon inhalation of OVA and LPS, two metabolic pathways Arg1 and iNOS, respectively are activated. The Arg1 pathway is triggered by type 2 cytokines (IL-4, IL-10, IL-13) released from OVA-specific Th2 effector cells. Concomitantly, LPS signaling via toll-like receptor 4 (TLR4) activates iNOS. The Arg1 pathway is down-regulated by L-hydroxy arginine (L-OH arginine), a product of iNOS pathway, whereas the iNOS pathway is down-modulated by consumption of L-arginine and polyamines, the end products of Arg1 pathway. Consequently, low concentrations of NO are produced which favor peroxynitrite (ONOO⁻) formation. NO at low concentrations and ONOO⁻ are pro-inflammatory molecules that increase airway inflammation. At the same time, immature dendritic cells (DCs) of the airways capture OVA, migrate to draining lymph nodes and undergo LPS-driven maturation. Mature DCs stimulate the proliferation of Th1 cells, which in turn, migrate to airways, where they inhibit allergic responses. In contrast, the expansion of Th1 cells is limited by NO derived from iNOS expressed by DCs upon LPS signaling through TLR4. Thus, it can be concluded that the effects of NO in asthma are critically dependent on its concentration produced under the influence of contaminations like LPS in the OVA.

**Genetics of iNOS and Asthma**

To substantiate further the role of iNOS in asthma, genetic studies involving asthmatic individuals in
Fig. 3—Gene structure of iNOS [iNOS is composed of twenty seven small exons and spans approximately 43 kb on chromosome 17q11. Various single nucleotide and the repeat polymorphisms of iNOS gene are marked] various populations have been undertaken. The gene for human iNOS lies within the C-C chemokine cluster region on chromosome 17q11.2-q12, where linkages with atopy and asthma have been reported\textsuperscript{37,48}. It comprises of 27 exons with the transcriptional start site in exon 2 and the stop codon in exon 27\textsuperscript{49}. There are several polymorphisms within the iNOS gene and its promoter\textsuperscript{50,51} (Fig. 3), several of them might be functional. From promoter variants, a highly polymorphic pentanucleotide (CCTTT)\textsuperscript{n} repeat approximately 2.5 kb upstream the transcription initiation site has been identified to affect the iNOS expression and shown to be associated with susceptibility to diseases like malaria, multiple sclerosis and rheumatoid arthritis\textsuperscript{51-53}. However, a limited number of genetic studies have been reported with atopy or asthma. The 14-repeat allele of CCTTT promoter polymorphism that affects promoter activity is inversely associated with atopy but not with asthma\textsuperscript{54}. In another report, no association of a biallelic repeat and asthma has been reported\textsuperscript{55}. Also, the promoter pentanucleotide repeat is not found to be associated with asthma or the exhaled NO concentration in another study\textsuperscript{56}.

Recently, three promoter polymorphisms and the 608Leu variant in exon 16 are found to be significantly associated with feather’s positivity and asthma severity respectively. Further, in haplotype analysis, the most common [-2447C/−1659C/−1026G/−0.7del/−277A/Ser608] haplotype is associated with a lower risk of asthma, when compared with the common haplotypes with frequency more than 5\%\textsuperscript{57}. In another study on 230 Indian families, a total of four repeats — a (CCTTT)n promoter repeat, a novel intron 2 (GT)n repeat (UniSTS:477297), an intron 4 (GT)n repeat (AFM311ZB1) and an intron 5 (CA)n repeat (D17S1878) have been identified in iNOS gene. A significant transmission distortion to the asthmatic probands is seen for allele with 15 repeats of the AFM311ZB1 ($P = 0.006$). This allele is also significantly associated with asthma severity ($P = 0.04$) and percentage blood eosinophils counts ($P = 0.0006$). Moreover, it is functionally correlated with high serum NO levels ($P = 0.006$). Similarly, the promoter repeat is found to be associated with serum total IgE ($P = 0.028$). Individuals carrying allele with 12 repeats of this (CCTTT)n repeat have high serum IgE ($P<0.0001$) as well as NO levels ($P = 0.03$)\textsuperscript{58}.

These results suggest that iNOS can play a role in atopic disorders and several polymorphisms in its gene may be important for asthma protection or susceptibility. However, the detailed analysis of the whole iNOS gene is required to elucidate the functional basis of the reported associations and to resolve the genetic role of iNOS in allergic diseases.

**Conclusion**

NO has pleotrophic role in the pathology of asthma, varying from an endogenous modulator of airway function to a pro-inflammatory and immunomodulatory mediator. Its actions may be determined by the concentrations generated (via c-NOS or iNOS) under specific circumstances, and the location and timing of synthesis\textsuperscript{59,60}. The bronchoprotective effects of NO in the asthma include airway smooth muscle relaxation and inhibition of smooth muscle proliferation. A deficiency of local NO, probably cNOS-derived NO may be responsible for the increased AHR in asthma. On the other hand, various animal experiments and genetic studies have shown that asthma is associated with locally increased production of NO generated from overexpressed iNOS which has detrimental effects within the Airways. NO has already evolved from the bench to the bedside and the non-invasive measurement of NO in exhaled air seems to accurately reflect inflammation in the airways and may be of value in
monitoring severity of airway diseases such as asthma. Understanding the exact molecular mechanism of NO action (i.e. involvement of second messenger, transcription factors etc.), selective and more potent NOS inhibitors and NO donors, comprehensive genetic studies as well as non-invasive clinical methods to assess NO biochemistry will lead to a better understanding of its deleterious and beneficial effects and developing novel treatments for asthma patients.

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