

## Role of endogenous nitric oxide in asthma

Endogenous nitric oxide (NO) is an ubiquitous signaling molecule with important regulatory functions such as regulation of blood pressure, neurotransmission, and host and immune defense. In the respiratory tract, NO is formed and released by various sources including endothelial and epithelial cells, nerves, airway smooth muscle, and inflammatory cells. Recent evidence suggests that endogenous NO is the neurotransmitter of the nonadrenergic noncholinergic inhibitory (iNANC) system, the only bronchorelaxant neural pathway of human airways. A number of studies also suggest that in some species epithelium-derived NO accounts for the functional bronchoprotective role of the so-called epithelium-derived relaxing factor. In human airways, endogenous NO counteracts the bronchoconstriction induced by pharmacologic stimuli such as bradykinin, histamine, and methacholine. On the basis of these and other observations, it is suggested that a reduced synthesis and/or activity of endogenous NO may contribute to the pathogenesis of airway hyperresponsiveness that characterizes asthma and other respiratory disorders. This short paper summarizes the activities of endogenous NO in the airways of experimental animals and man, and discusses the evidence supporting the view that NO confers bronchoprotection.

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Key words: asthma; nitric oxide; smooth muscle.

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It is now well established that endogenous nitric oxide (NO), first identified in 1986 as the endothelium-derived relaxing factor, has a key role in a number of physiologic processes including arterial blood flow and pressure regulation, neurotransmission, host defense, and immune and inflammatory responses (1, 2). In the respiratory tract of various mammalian species, NO is released by a variety of cells such as nerves, endothelial and epithelial cells, vascular and airway smooth muscle, and inflammatory cells. Over the last 10 years, it has become evident that NO is involved in the regulation of pulmonary functions, and that some respiratory disorders might be related to either a defect or an excess of NO formation and/or action at cellular sites. Both these aspects of NO activity seem to overlap and play an important role in the pathogenesis of bronchial asthma. High levels of NO closely related to the degree of airway inflammation have been detected in the exhaled air of asthmatic patients (3, 4). These high levels are probably due to the increased production of NO from inflammatory cells and might be involved in the generation and maintenance of the airway inflammatory response. On the other hand, NO itself is a bronchodilator and is now considered the neurotransmitter of the nonadrenergic noncholinergic inhibitory system (i-NANC), which is the only neural relaxant pathway in human airways. Furthermore, several lines of evidence suggest that in the airways of asthmatic patients the release of NO

might be a physiologic mechanism counteracting the bronchoconstriction caused by various stimuli. These conflicting biologic aspects of endogenous NO in asthma have greatly intrigued researchers over the last decade. This short paper focuses on the putative bronchoprotective role of NO in the airways of asthmatic subjects.

### Synthesis and cellular effects of NO

NO is a diatomic free radical derived from the guanidino nitrogen of the semiessential amino acid L-arginine by the action of the enzyme NO synthase (NOS). In the presence of a number of cofactors, L-arginine undergoes a five-electron oxidation to release the amino acid L-citrulline and NO (5). NO is a short-lived (0.1–0.5 s) and highly reactive molecule which is rapidly converted to nitrogen dioxide, nitrite, and nitrate (6). In the respiratory system, in the presence of high levels of the superoxide anion, NO decomposes in the peroxynitrite anion, a strong oxidant agent which facilitates membrane lipid peroxidation, thus contributing to lung damage (7). In the blood, NO, due to its high affinity with the heme group, binds to and is rapidly inactivated by hemoglobin.

NOS exists in three established isoforms: two constitutive isoforms (ncNOS or NOS I, originally found in neurons, and ecNOS, NOS III, expressed in endothelial cells) and one inducible isoform (iNOS,

NOS II, first identified in murine macrophages, but also inducible in many other cell types). All these NOS isoforms can produce NO from L-arginine (8). Constitutively expressed NOS isoforms are calcium/calmoduline-dependent proteins responsible for the release of small amounts of NO within seconds or minutes after receptor stimulation with agonists. NOS II is a transcriptionally regulated enzyme whose activity is induced by a variety of stimuli including cytokines, lipopolysaccharides, and, in airways, some environmental pollutants and allergens (9–11). In contrast to NOS I and NOS III, the release of NO by NOS II takes several hours and continues for days after exposure to the activating stimulus. Glucocorticoids and some cytokines such as transforming growth factor- $\beta$  inhibit the induction of NOS II but have little or no effect on NOS I and NOS III activity. However, recent evidence indicates that these two isoforms are also subject to expressional regulation. Therefore, in view of their important biologic functions, expressional changes of NOS I and NOS III may have important pathophysiologic consequences.

The intracellular pathway initiated by NO consists of the activation of soluble guanylyl cyclase (sGC), leading to an increased synthesis of the intracellular messenger cyclic guanosine monophosphate (cGMP) promoting smooth-muscle relaxation via the activation of intracellular kinase and/or opening of membrane  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels (12–14).

A number of analogs of L-arginine, by acting as false substrates of NOS, are able to reduce NO production, thus providing important tools to investigate the pathophysiologic roles of NO.  $\text{N}^{\text{G}}$ -L-monomethyl-arginine (L-NMMA) and  $\text{N}^{\text{G}}$ -nitro-L-arginine methyl ester (L-NAME) are the most commonly used inhibitors (15). Their use has helped in disclosing numerous NO functions.

### NO and airway smooth muscle

Several studies suggest that inhalation of exogenous NO can counteract methacholine-induced bronchoconstriction in experimental animals (16, 17). Despite little or no effect on resting bronchomotor tone in healthy subjects, inhaled NO causes a weak bronchodilation in asthmatics and mitigates the bronchoconstriction induced by methacholine in man (18, 19). These observations are in agreement with previous *in vitro* studies showing the bronchorelaxant properties of the NO donors glyceryl trinitrate and sodium nitropruside. The involvement of endogenous NO in the modulation of bronchial tone was first suggested by studies in isolated guinea-pig trachea showing that the electrical field stimulation (EFS)-induced iNANC relaxant response was partially reversed by incubation with the NOS inhibitors L-NAME or L-NMMA, thus

indicating that NO mediates, at least in part, iNANC neurotransmission in this experimental animal model (20–22).

The importance of NO as a neurotransmitter of the iNANC system has been subsequently demonstrated in isolated human airways (Table 1) (23). The presence of the iNANC neural system, which is greater in central and decreases progressively in distal human airways (24, 25), provides the only endogenous bronchodilator pathway of this species. Therefore, the notion that in isolated human airways the iNANC response is solely mediated by NO assumes great functional relevance. In addition, in human airways, the iNANC functional response is associated with a concurrent increase in cGMP content in smooth-muscle cells (26). Pre-treatment of both guinea-pig and human isolated airways with NOS inhibitors significantly increases EFS-evoked cholinergic contractions (27, 28). Since NOS inhibitors do not affect acetylcholine (ACh) release from nerve terminals, it is likely that NO modulates the cholinergic neural response by functional antagonism of ACh in the smooth-muscle cell.

In the respiratory tract, the presence of NOS-immunoreactive nerves has been well documented in bronchial vessels, airway smooth muscle, and submucosal glands (29–31). In man, the density of these nerve fibers is higher in the central and decreases in the peripheral airways (25, 30). This is in agreement with the distribution of iNANC functional responses (25). Since the expression of NOS is present in parasympathetic nerves, it is possible that NO is coreleased with ACh and other neurotransmitters, such as vasoactive intestinal peptide, to modulate cholinergic responses. Interestingly, it has also been shown that in guinea-pig trachea endogenous NO, modulating responsiveness to histamine, can be released from sympathetic nerves (32). The recent observation that in cats the nonadrenergic bronchodilation induced by capsaicin is suppressed by L-NAME provides the first *in vivo* evidence that the iNANC response is mediated by NO.

Consistent with this observation is the hypothesis that the iNANC response might be somehow impaired as a consequence of airway inflammation observed in disease states such as asthma. This would enhance the bronchoconstrictive response to various stimuli. In addition to enhanced bronchoconstriction, the reduced iNANC activity could play other roles in asthma. It has

Table 1. Nitric oxide (NO) meets criteria for iNANC neurotransmitter

- 1) Synthesis and release of NO on nerve activation
- 2) Release by  $\text{Ca}^{2+}$ -dependent mechanism
- 3) Action on local cells by diffusion
- 4) Mechanisms for inactivation exist
- 5) Mimicking action on exogenous administration
- 6) NO antagonists inhibit iNANC activity
- 7) NO-donor compounds mimic iNANC activity

recently been suggested that circadian variations of the iNANC response may contribute to overnight bronchoconstriction in patients with nocturnal asthma (33, 34). It is also noteworthy that the iNANC response is impaired in the airways of patients with cystic fibrosis, in which there is an intense neutrophilic inflammation of the airways (35).

In experimental animals, the iNANC-mediated relaxation is blocked by tetrodotoxin, indicating that this phenomenon is originated by a neural pathway. It is also possible, however, that the airway epithelium participates in these events as an effector organ. In fact, in some studies, a nonneural component of the relaxant response to EFS resistant to tetrodotoxin has been found. This nonneural component is absent in guinea-pig tracheal preparation denuded of epithelium, suggesting the importance of the epithelial layer in the genesis of this phenomenon. Epithelial cells are a major source of NO in man and other mammalian species. In the airways of these species, the removal of the respiratory epithelium increases the contractile response of the underlying smooth muscle. This is due not simply to the lack of a mechanical barrier but more likely to the lack of a putative relaxing factor, which is normally secreted by the airway epithelium, and which counteracts bronchoconstriction to various spasmogens. A growing body of evidence suggests that endogenous NO accounts for the functional effects of epithelium-derived relaxing factor. In guinea-pig airways, despite the lack of any effect on basal bronchial tone, epithelial-derived NO reduces contractile response to cholinergic agonists, histamine, KCl, and bradykinin (36–39). We have recently found that in intact, but not in epithelium-denuded, bovine trachealis, NOS inhibition enhances the contractile response to ACh and histamine, but has no effect on the response to 5-hydroxytryptamine (40). Since epithelial shedding is present in the airways of asthmatic patients, it has been proposed that the lack of epithelium-derived NO, in addition to the direct exposure of smooth muscle to exogenous stimuli, may contribute to the increased airway responsiveness in these patients.

#### NO and bronchial smooth muscle *in vivo*

The role of endogenous NO in both animals and man *in vivo* has been extensively investigated. In the guinea pig, the inhalation of bradykinin causes bronchoconstriction by various mechanisms. This bronchoconstriction is greatly increased by the inhibition of the L-arginine NO synthase pathway, suggesting that inhaled bradykinin releases NO, which in turn relaxes airway smooth muscle (41, 42). *In vivo* studies suggest that in guinea-pig airways NO is not released from neural elements. This is further supported by data showing that bradykinin-induced NO production occurs at the

respiratory epithelium level *in vitro*. A similar protective effect of endogenous NO against bradykinin-induced bronchoconstriction has also been recently reported in the rat. Furthermore, it has recently been shown that in the guinea pig endogenous NO attenuates the bronchoconstriction induced by inhalation of citric acid, which is mediated by the release of tachykinins from sensory nerve endings (43).

The role of endogenous NO has been widely investigated in man. The inhalation of L-NMMA significantly enhances bronchoconstriction to inhaled bradykinin in mildly asthmatic subjects but has no effect on their basal bronchial tone. There is also evidence that in man bradykinin-induced bronchoconstriction is mediated by neural mechanisms such as the release of ACh from postganglionic cholinergic nerves and tachykinins from sensory nerves. It is therefore possible that NO is coreleased by the action of bradykinin on neural structures (cholinergic nerves, sensory nerves, and iNANC nerves), although the release of NO from nonneural cell types and local inflammatory cells cannot be excluded. Another important consideration is that inhibition of the NO synthase pathway does not potentiate bradykinin-induced bronchoconstriction in patients with severe asthma, indicating that the regulatory role of endogenous NO may be less important in these patients (44). The reason for this reduced importance of NO in severe asthma is far from being clear. One can speculate, however, that in these patients the extended epithelial damage, resulting from chronic airway inflammation, may lead to a reduction or absence of cNOS activity; thus, loss of bronchoprotective NO could be an important mechanism associated with more severe asthmatic conditions. In asthmatic patients, inhalation of NOS inhibitors also potentiates bronchoconstriction induced by histamine, methacholine, adenosine 5'-monophosphate, and inflammatory mediators such as leukotriene D<sub>4</sub> (45, 46). The bronchoprotective role of endogenous NO in asthma has also been recently suggested by the finding of low concentrations of the NO-adduct compound S-nitrosothiols in the airways of children with near fatal asthma (47). Since S-nitrosothiols are naturally occurring bronchodilators, this finding confirms the role of NO and NO products in regulating the airway response in asthma.

Despite the widely recognized importance of endogenous NO in asthma, the exact mechanism underlying NO bronchoprotection is not fully understood. *In vitro* studies on guinea-pig trachea suggest that the increase in airway responsiveness induced by NOS inhibition might be due to a shift from bronchodilator cyclooxygenase products to proinflammatory and bronchoconstrictor lipoxygenase products, notably leukotrienes (48). However, so far, this puzzling assumption has never been confirmed in human models.

Finally, the present evidence suggests that in some animal species endogenous NO plays an important regulatory role in acute allergic bronchoconstriction. In a guinea-pig model of allergic asthma, it has been shown that endogenous NO modulates the bronchoconstriction caused by inhaled allergens as well as allergen-induced airways hyperreactivity to histamine (49–51). Endogenous NO derived from the neural NOS isoform has also been recognized as an important factor in controlling the inducibility of airway hyperresponsiveness in a murine model of allergic asthma (52). Surprisingly, these findings have never been confirmed in man. Conflicting with the hypothesis that endogenous NO is bronchoprotective in clinical asthma is the observation that pharmacologic inhibition of NOS activity does not affect either the early or the late asthmatic response to inhaled allergen in patients with mildly allergic asthma (46).

### Conclusion

Despite recent advances, airway tone regulation and responsiveness in asthma are rather complex phenomena whose origin is largely unknown. Increased levels

and/or activity of a number of inflammatory mediators have been suggested as important factors involved in the pathogenesis of the exaggerated airway response in asthma. Another hypothesis, however, is the existence of putative bronchoprotective factors, whose impaired activity may contribute to the pathogenesis of asthma and airway hyperresponsiveness. A number of experimental studies have provided evidence that the L-arginine NO synthase pathway is involved in the regulation of airway responsiveness. Taken together, *in vitro* and *in vivo* studies strongly suggest that in human and animal airways endogenous NO might be released from several neural and nonneural sources in response to a variety of pharmacologic bronchoconstrictive stimuli through mechanisms which are largely undetermined. Despite a large body of information supporting the bronchoprotective effect of NO in asthmatic airways, the pathophysiologic relevance of the NO pathway still remains to be determined. One possible speculation is that a better understanding of such mechanisms may represent an important background for the development of new therapeutic options in the treatment of bronchial asthma.

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