OCCASIONAL REVIEW

Multiple roles of nitric oxide in the airways

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Nitric oxide is endogenously released in the airways by nitric oxide synthase. Functionally, two isoforms of this enzyme exist: constitutive and inducible. The former seems to protect airways from excessive bronchoconstriction while the latter has a modulatory role in inflammatory disorders of the airways such as asthma. This review explores the physiological and pathophysiological role of endogenous nitric oxide in the airways, and the clinical aspects of monitoring nitric oxide in exhaled air of patients with respiratory disease.

Nitric oxide (NO) has long been considered an atmospheric pollutant present in smog and cigarette smoke, a destroyer of ozone, and a precursor of acid rain.[1] Since the discovery in 1987 that NO was similar to endothelium derived relaxing factor (EDRF),[2] its importance in the regulation of body functions, including the respiratory tract, has become apparent.

NO has a short half life (1–5 s) and one unpaired electron, making it a free radical that reacts with other molecules such as oxygen, superoxide radicals, or transition metals (such as iron bound within haemoproteins). It is an ubiquitous messenger molecule that regulates various biological functions—either at low concentrations as a signal in many physiological processes including blood flow regulation, platelet reactivity, non-adrenergic non-cholinergic (NANC) neurotransmission and memory, or at high concentrations as cytotoxic and cytostatic defensive mechanisms against tumours and pathogens.[3] Increasing evidence also points to an important role for NO in the regulation of pulmonary function and in pulmonary disease.[4–6] Moreover, NO has been detected in exhaled air of animals and humans[7] and is increased in various inflammatory diseases of the airways such as bronchial asthma.[8]

CELLULAR SOURCE AND BIOSYNTHESIS OF NO

In the respiratory tract NO is produced by a wide variety of cell types including epithelial cells, airway nerves, inflammatory cells (macrophages, neutrophils, mast cells), and vascular endothelial cells.[4] Given the many locations from which NO may be produced, its precise physiological activity in any given part of the lung may be difficult to determine. The activity of NO typically depends on many local factors including the amount and activity of the enzymes responsible for producing NO, the level of oxidant stress, and its rate of uptake by antioxidant molecules such as haemoglobin and glutathione. NO is generated via a five-electron oxidation of a terminal guanidinium nitrogen on the amino acid L-arginine. The reaction is both oxygen-dependent and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and yields the coproduct L-citrulline in addition to NO in a 1:1 stoichiometry.[9] This reaction is catalysed by NO synthase (NOS) which exists in three distinct isoforms: (1) constitutive neural NOS (NOS-I or nNOS); (2) inducible NOS (NOS-II or iNOS); and (3) constitutive endothelial NOS (NOS-III or eNOS). The three distinct isoforms of NOS have been identified by protein purification and molecular cloning. nNOS, iNOS, and eNOS are products of distinct genes located on different human chromosomes (12, 17, and 7 chromosomes, respectively), each with a characteristic pattern of tissue specific expression.[10]

NOS is structurally divided into two major domains, the reductase and oxygenase domains.[11] The C-terminal region, termed the reductase domain, possesses consensus sequences for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and NADPH binding sites, and exhibits close sequence homology to another mammalian enzyme, cytochrome P-450 reductase. The N-terminal region, termed the oxygenase domain, is thought to function as heme, tetrahydrobiopterin (H_4B), and L-arginine binding sites. NADPH acts as the source of electrons for oxygen activation and substrate oxidation. It is also believed that FAD and FMN play a role in shuttling electrons from NADPH to the iron heme. Thus, the heme component of NOS represents the catalytic centre, responsible for binding and reducing molecular oxygen and subsequent oxidation of substrate. Linking the reductase and oxygenase domains is a consensus sequence representative of a calmodulin binding site. The function of calmodulin is to transfer electrons between flavins and the heme moiety and to couple the reductase and oxygenase domains.[12]

Functionally, NOS exists in constitutive (cNOS) and inducible (iNOS) forms.[13] cNOS is expressed in platelets and in neuronal, epithelial, and endothelial cells. This enzyme is Ca^{2+}- and calmodulin-dependent and releases, within seconds, fM or pM concentrations of NO upon receptor stimulation by agonists such as acetylcholine and bradykinin.[13] iNOS expression has been described in macrophages, neutrophils, hepatocytes and epithelial, mesangial, endothelial and vascular smooth muscle cells. This isoform is regulated at a pretranslational level and can be induced by proinflammatory cytokines such as tumour necrosis factor (TNF)-α, interferon (INF)-γ and interleukin(IL)-1β.[14] iNOS releases large quantities (nM concentrations) of proinflammatory NO several hours after exposure and may continue in a sustained manner (hours or days).

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The subcellular localisation of the three NOS isoforms is still controversial. nNOS and iNOS have been characterised as soluble (cytosolic) proteins while eNOS is targeted to the particulate subcellular fraction, specifically to plasmalemmal caveolae (small invaginations in the plasma membrane characterised by the presence of the transmembrane protein caveolin). In endothelial cells it has been shown that the association between eNOS and caveolin suppresses eNOS activity. After agonist activation the increase in $[Ca^{2+}_i]$ promotes calmodulin binding to eNOS and the dissociation of caveolin from eNOS. The eNOS-calmodulin complex synthesises NO until $[Ca^{2+}_i]$ decreases and then the inhibitory eNOS-caveolin complex reforms.[15]

NO AND MECHANISMS OF ACTION

NO diffuses rapidly from the point of synthesis, can permeate cell membranes, interacts with intracellular molecular sites within both generating and target cells, and has intrinsic instability—all properties that eliminate the need for extracellular NO receptors or targeted NO degradation. The best characterised target site for NO is the iron bound in the heme component of soluble guanylate cyclase, stimulating conversion of GTP to cGMP and mediating the biological effects attributed to cNOS derived NO.[16] In addition, NO mediates other actions that are independent of guanylate cyclase and cGMP. The high levels of NO released by iNOS act as an immune effector molecule killing tumour cells,[17] halting viral replication,[18] and eliminating various pathogens. In fact, NO has been reported to inhibit the growth of or to kill a number of fungi, parasites, and bacteria including *Mycobacterium tuberculosis*.[19] This mechanism may involve inhibition of DNA synthesis by inactivation of ribonucleotide reductase and by direct deamination of DNA.[20 21]

The interaction of NO with many molecular targets represents an important pathway for its breakdown and inactivation. The most important interaction is with superoxide anion (O_2^-) to yield peroxynitrite anion ($ONOO^-$) which is a potent cytotoxic molecule.[22] Another mechanism of inactivation which appears to be important in vivo involves the reaction of NO with molecular oxygen to form nitrite (NO_2^-) which, in the presence of haemoproteins (haemoglobin), is oxidised further to nitrate (NO_3^-).[23] NO has also been shown to react with thiol-containing proteins such as albumin and tissue plasminogen activator to form S-nitrosothiols which may act as storage or carrier forms of NO.[24]

METHODS OF NO DETECTION

The presence of NO in biological systems is usually inferred from its physiological effects such as increased cGMP concentration, production of citrulline, or change in functional response produced by NOS inhibitors. These methods provide indirect information about NO production and have different degrees of specificity. Direct measurement of NO is difficult, both because of the small amounts present (usually ≤nM concentrations) and the lability of NO in the presence of oxygen. NO is currently measured by spectroscopic and electroanalytical methods.

Spectroscopic methods include chemiluminescence,[25] ultraviolet (UV) visible spectroscopy,[26] and electron paramagnetic resonance.[27] Chemiluminescence assay is based on the measurement of the intensity of the fluorescent radiation emitted after chemical oxidation of NO by ozone using a sensitive photomultiplier tube (PMT). The product of this reaction (NO_2^*) emits a photon, and the total number of photons produced is proportional to the NO concentration.[28] The UV visible spectroscopic method for NO determination is based on the Griess reagent which is a mixture of sulfanilic acid and N-(1-naphthyl) ethylenediamine. N-(1-naphthyl) ethylenediamine reacts with NO, and a spectrum of the product of this reaction shows a band at 548 nm. The absorbance of this peak is proportional to the NO concentration.[28] Another UV visible spectroscopic method is based on the rapid oxidation of reduced haemoglobin (Fe^{2+}) to methaemoglobin (Fe^{3+}) by NO. NO is detected by observing the characteristic shift in the Soret absorbance peak of haemoglobin from 433 nm to 406 nm.[28] Electron paramagnetic resonance (EPR) assay can be used to monitor molecules with unpaired electrons including radicals such as NO. In fact, NO is a gaseous paramagnetic molecule with the unpaired electron in the π orbital. In this method, a spin trap such as haemoglobin is used to stabilise NO. Spin traps are compounds that interact with unstable radicals, producing a more stable adduct such as nitrosylhaemoglobin (NO-Hb) that can be detected by EPR.[28]

Electrochemical methods of NO determination offer several features that are not available by spectroscopy. These methods are based on the electrochemical oxidation of NO on solid electrodes.[29] If the current generated during its oxidation is linearly proportional to the concentration, the oxidation current can be used as an analytical signal. This current can be measured either in the amperometric or voltametric mode. Two sensors have been developed for the electrochemical measurement of NO.[29] One is based on the electrochemical oxidation of NO on a platinum electrode (the classical Clark probe for detection of oxygen), and the other is based on the electrochemical oxidation of NO on polymeric porphyrin (porphyrinic sensor). The oxidation of NO on solid electrodes involves an electrochemical reaction followed by a chemical reaction. The first electrochemical step is a one electron transfer from an NO molecule to the electrode resulting in the formation of nitrosonium (NO^+). The second step is the chemical conversion of NO^+ to nitrite (NO_2^-) in the presence of OH^-. The porphyrinic electrode is not able to differentiate nitrite produced electrochemically from that derived from NO oxidation, so a barrier has to be placed between the electrode and the analyte to prevent access of nitrite to the electrode surface. This has been achieved in the porphyrinic sensor by depositing a layer of cation exchanger (Nafion) which repels negatively charged species such as NO_2^-.

The high sensitivity and fast response of the electrochemical method permit in situ monitoring of the kinetics of NO levels on the surface of tissue or cell cultures. However, to measure NO levels in intact organs such as the lung, where the kinetic information is not the target, spectroscopic methods are preferred.

PHYSIOLOGICAL ROLE OF NO IN THE AIRWAYS

NO and bronchodilation

Since the discovery that NO containing vasodilators such as glyceryl trinitrate and sodium nitroprusside induce relaxation of the isolated airway smooth muscle, activate guanylate cyclase, and raise cGMP,[30] the ability of inhaled NO to provoke a bronchodilator effect in animals and man has been studied. In anaesthetised guinea pigs methacholine induced bronchoconstriction is reduced by inhaled NO in a concentration dependent manner from 5 ppm to 300 ppm.[31] In addition, a high concentration of NO (300 ppm) causes a small degree of baseline bronchodilation. In anaesthetised and mechanically ventilated rabbits 80 ppm NO added to the inspired gas prevented an increase in resistance to nebulised methacholine.[32] However, no effect was seen on compliance, suggesting that NO prevented the contraction of the larger airways more than the small airways.[32] Inhaled NO at a concentration of 80 ppm has no effect in normal individuals and in patients with chronic obstructive pulmonary disease (COPD), but has a small bronchodilator effect in asthmatic patients.[33] There is evidence that, in addition to guanylyl cyclase activation, NO relaxes bronchial smooth muscle by another mechanism.[34] NO is involved in the metabolic pathway of thiol to form nitrosothiols (RS-NO).[35] RS-NO are

present in the airways of normal subjects and have a substantially greater half life than NO at physiological concentrations of oxygen.[35] RS-NO possess potent bronchodilator activity, are not dependent on the cGMP pathway,[36] and are present in concentrations sufficient to influence airway tone.[35] It has recently been found that severe asthma is associated with low concentrations of RS-NO in the airway, suggesting that the deficiency of such an endogenous bronchodilator mechanism is due to an accelerated degradation in the lungs of severe asthmatic patients, contributing to severe and refractory bronchospasm.[37]

Besides the classical cholinergic and adrenergic systems driving the bronchomotor tone, a non-adrenergic non-cholinergic (NANC) neural system exists in the airways of animals and humans which mediates contraction (excitatory NANC (eNANC)) or relaxation (inhibitory NANC (iNANC)).[38 39] Evidence obtained in recent years indicates that NO acts as a neurotransmitter of iNANC, and nitrergic neurotransmission has been shown not only in the airways but also in the gut, bladder, and reproductive organs.[4] Immunocytochemical staining of nNOS shows localisation in the nerves of guinea pig and human airways. NOS immunoreactive nerves appear to supply airway vessels, airway smooth muscle, and the lamina propria.[40] NOS immunoreactive neurones are found in parasympathetic ganglia and in sympathetic and sensory ganglia (more in jugular than in nodose) supplying the airways,[40 41] and are more prominent in proximal than in distal airways.[42] NO is released from peripheral nerves by nNOS and is activated by calcium entry when the nerve is depolarised.[39] It mediates approximately half the iNANC (relaxant) response in guinea pig trachea in vitro, and the neuropeptide vasoactive intestinal peptide (VIP) should be involved in the second part of the iNANC relaxant response.[43] In man the iNANC response in central and peripheral airways is completely mediated by NO.[44 45] Furthermore, inhibition of NOS potentiates cholinergic neural bronchoconstriction without influencing neural acetylcholine release,[46 47] suggesting that nNOS derived NO is a functional antagonist to excitatory cholinergic pathway acting postjunctionally and not at the prejunctional level.[47] It has also been observed that endogenous NO released in association with nerve stimulation regulates the magnitude of the eNANC response in guinea pig airways.[48] Neural NO induced relaxation is impaired in allergic inflammation of the airway, even though no change in nNOS expression has been reported, indicating altered neural NOS activity in the presence of allergic inflammation leading to exacerbation of asthma.[49]

NO and bronchoprotection

Airway hyperresponsiveness (AHR), which is the main feature of asthma, is defined as an increase in the ease and degree of airway narrowing in response to bronchoconstrictor stimuli. Several reports have shown the ability of endogenous NO to influence baseline AHR induced by different mediators in animal models. In 1993 Nijkamp *et al* described the ability of NOS inhibitors in guinea pigs to potentiate either bronchoconstriction induced by histamine in vivo or the histamine concentration dependent contraction of tracheal tubes in vitro,[50] suggesting a modulator role for endogenous NO in AHR. Furthermore, Ricciardolo *et al* found an NO dependent modulation of bronchoconstriction induced by bradykinin, citric acid, tachykinin NK_1 selective agonist, and protease activated receptor 2 in guinea pigs.[51–54] Different groups of investigators have shown that acute bronchoconstriction induced by allergen inhalation is potentiated by NOS inhibitors in sensitised guinea pigs, suggesting modulation by endogenous protective NO on the early asthmatic reaction in an animal model.[55–57] Intraluminal perfusion of tracheal tube preparations has shown that bradykinin, endothelin-1, substance P, adenosine, and calcitonin gene related peptide applied to the inside of intact tracheal tubes provoke concentration dependent relaxation.[58–63] The relaxation is reversed into contractions (or contractions are markedly potentiated) by NOS inhibitors, indicating that the relaxant effect in the airways is mediated by the release of endogenous NO.[58–63] This effect was mimicked by removal of airway epithelium, suggesting that airway epithelium releases NO which counteracts smooth muscle contraction induced by different spasmogens. These striking results indicate the functional importance of airway epithelium in AHR, which is not considered as a physical protective barrier between constrictors and smooth muscle but as a modulator of bronchomotor tone via the release of relaxant substances (so-called epithelium derived relaxing factors). A further study showed that the electrochemical detection of bradykinin induced NO release in guinea pig airways was fast (about 2 s), mainly dependent on the epithelium, and absent in Ca^{2+} free medium, which suggests that a Ca^{2+} dependent cNOS pathway is involved in the endogenous release of bronchoprotective NO.[64] The subsequent step of epithelial derived NO release is the paracrine effect on airway smooth muscle that is dependent on cGMP increase in the effector cell. In fact, it has been shown that bradykinin significantly raises cGMP levels in guinea pig airways and that this effect is blocked by pretreatment with NOS inhibitors and in epithelium denuded preparations, suggesting that cGMP is the final mediator of bronchoprotection dependent on epithelium derived NO.[58]

Allergen and viral infection also induce AHR in animal and human asthma.[65 66] Recent in vitro and in vivo studies have shown that the increased AHR induced by allergen (6 hours after exposure) is not potentiated by pretreatment with NOS inhibitors,[67–69] and that virus induced AHR is completely blocked by low doses of inhaled L-arginine,[70] suggesting that enhanced AHR is dependent on the deficiency in endogenous release of protective NO. More specifically, the authors proposed that a deficiency in cNOS derived NO contributes to the increased AHR after the early response (EAR) to allergen (4–6 hours) and a recovery in iNOS derived NO production aids the reversal of AHR after the late response (LAR, 24–48 hours) in guinea pigs, as shown by the lack of effect on AHR to histamine after EAR and by a significant potentiation of the partially reduced AHR to histamine after the LAR induced by inhalation of the specific iNOS inhibitor aminoguanidine.[71] In line with this evidence, Toward and Broadley found that exposure of guinea pigs to inhaled lipopolysaccharide (LPS) initially inhibited NO synthesis and the reduced NO levels coincided with the period of increased AHR to histamine (1 hour after exposure).[72] In contrast, 48 hours after LPS exposure the bronchoconstrictor response to histamine was attenuated (airway hyporesponsiveness) in association with increased levels of NO metabolites in the bronchoalveolar lavage fluid, suggesting renewed NO synthesis probably resulting from cytokine induced NF-κB activation of iNOS gene[73] with relaxant effect. Finally, it has also been hypothesised that the deficiency of cNOS derived NO in allergen induced AHR after EAR in guinea pigs may be due to limitation of substrate for cNOS activity induced by eosinophil derived polycationic peptides such as major basic protein.[74]

On the basis of these studies, clinical researchers have investigated the ability of endogenous NO to affect excitatory airway responses in asthma. Ricciardolo *et al*[75] performed a randomised, double blind, placebo controlled study of the effect of NOS inhibition in bradykinin induced asthma. They found potentiation of bradykinin and methacholine induced AHR after pretreatment with the NOS inhibitor, suggesting a bronchoprotective role for endogenous NO in mild asthma. Furthermore, they found that this potentiation was much greater in AHR to bradykinin than to methacholine, indicating that a mediator specific response is involved. In a further study the same group found impaired NOS inhibition of AHR to bradykinin in patients with severe asthma, possibly due to the reduction or absence of cNOS in the airway of these

patients.[76] Following these observations, it has also been found that severe asthmatics treated with higher dose of corticosteroids than in the previous study are less hyperresponsive to bradykinin, but that pretreatment with NOS inhibitor markedly enhanced AHR to bradykinin as in patients with mild asthma.[77] This suggests that high doses of corticosteroids renew cNOS activity by suppressing iNOS expression. Finally, significant potentiation of NOS inhibitors has been found in AHR to AMP and histamine, but not to allergen induced bronchoconstriction, in patients with asthma.[78 79]

The possible involvement of the eNOS gene as the genetic basis of bronchial asthma has been investigated by examining whether there is an association between bronchial asthma and polymorphisms of the eNOS gene. Lee *et al*[80] found that the distribution of one genotype (bb) of eNOS was significantly higher in the asthma group than in the control population, but the eNOS genotype distribution did not differ significantly among groups of patients with asthma of different severity. These results suggest that polymorphisms of the eNOS gene may be associated with the development of asthma, but that the severity of asthma is not influenced by polymorphisms of the eNOS gene.

NO and vessels

Nitric oxide can account for the biological activity of EDRF and is involved in the regulation of vascular tone.[81] The release of NO from endothelial cells in the pulmonary circulation appears to regulate vascular basal tone and counteract hypoxic vasoconstriction.[82] Furthermore, NO release is apparently decreased in chronic hypoxia.[83] The eNOS isoform is present in the endothelium of pulmonary vessels of healthy subjects but its expression is downregulated in patients with primary pulmonary hypertension,[84] which suggests that pulmonary vasoconstriction and an increased smooth muscle layer in the pulmonary vessels, which are features of this disease, are due to impaired expression of eNOS. Impaired release of endothelium derived NO has also been observed from pulmonary vessels of patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis.[85] Moreover, isolated pulmonary arteries of patients undergoing heart-lung transplantation for end stage chronic lung disease have impaired endothelium dependent relaxation.[86] It has recently been shown that overproduction of eNOS derived NO can inhibit, not only the increase in right ventricular systolic pressure associated with pulmonary hypertension, but also remodelling of the pulmonary vasculature and right ventricular hypertrophy induced by chronic hypoxia, suggesting a potential therapeutic role of overexpression of the eNOS pathway in pulmonary vascular endothelium.[87]

Endogenous NO regulates basal bronchial vascular tone and exogenous NO accounts for most of the bronchial vasodilation observed after inhalation of cigarette smoke.[88] The airway vasculature has also been shown to dilate in vivo when animals are ventilated with NO.[89] Finally, endogenous endothelial NO significantly influences acetylcholine induced bronchovascular dilation,[90] but not vagally induced bronchial vascular dilation, in sheep.[91]

Conflicting results have been reported on the role of endogenous NO in vascular permeability.[92] A recent study in guinea pigs showed that NOS inhibitors inhibit airway microvascular plasma leakage induced by substance P and leukotriene D4 (LTD4) but not by histamine, suggesting that endogenous NO plays an important role in plasma extravasation induced by some inflammatory mediators.[93] The authors also showed that the rise in plasma extravasation induced by substance P and LTD4 is increased via endogenous NO in the trachea and main bronchi, but not in the intrapulmonary airways, suggesting differential regulation of transvascular protein flux in anatomically different parts of the airway microvasculature. The inhibition of substance P induced plasma extravasation by NOS inhibitor is possibly due to the vasoconstriction of perfused vessels and the subsequent decrease in local blood flow at the leaky site. These results may be explained by the reduction of bronchial blood flow by NOS inhibitors. Further studies examining blood flow through individual microvascular beds would permit more information on the precise role of endogenous NO on this important aspect of airway microcirculation relevant to diseases such as asthma.

NO and airway secretions

To determine whether NO regulates mucus secretion from airway submucosal glands, which are the main source of human secretion,[94] the effects of NOS inhibitors (L-NAME and L-NMMA) on mucus glycoprotein secretion has been determined by measuring trichloroacetic acid precipitable [3H]-glycoconjugates from human airway explants and isolated submucosal glands.[95] NOS inhibitors did not alter mucus glycoprotein secretion tonically, but significantly inhibited both methacholine and bradykinin induced secretion from isolated glands. Furthermore, the NO generator isosorbide dinitrate induced a significant increase in secretion. This finding suggests that endogenous NO has stimulatory activity in airway submucosal gland secretion.[95] Other secretagogues such as platelet activating factor, histamine, and TNF-α enhance release of mucin by guinea pig tracheal epithelial cells, but the stimulatory effect of each is inhibited by prior co-incubation of the cells with a competitive inhibitor of NOS, indicating that these mediators provoke mucin secretion via a mechanism involving intracellular production of NO as a critical signalling molecule.[96]

NOS inhibitors are also able to slow ciliary beat frequency of bovine airway epithelial cells pre-stimulated with isoproterenol, bradykinin, and substance P, and this effect is completely reversed by L-arginine, an NO precursor, which indicates that an NO-dependent mechanism upregulates ciliary motility in response to stimulation.[97] Ciliary motility is an important host defence mechanism of airway epithelium and is enhanced by the iNOS inducing alveolar macrophage derived cytokines such as TNF-α and IL-1β.[98] The stimulatory effect of TNF-α and IL-1β on cilia is inhibited by L-NMMA and restored by the addition of L-arginine, suggesting an involvement of the iNOS pathway in the regulation of ciliary motility.[98]

Abnormal electrolyte transport produces changes in airway surface liquid volume and composition, inhibits mucociliary clearance, and leads to chronic infection of the airways, as in cystic fibrosis. Modulation of ion channels by NO has recently been found to be a significant determinant of ion channel function.[99] Nitric oxide activates both apical anion channels and basolateral potassium channels via a cGMP-dependent pathway.[100] Thus, NO is a physiological regulator of transepithelial ion movement and changes in its generation and activity may play an important part in the pathogenesis of lung disorders characterised by hypersecretion of airway surface liquid.

PATHOPHYSIOLOGICAL ROLE OF NO

NO and airway inflammation

Increased production of endogenous NO results in a long term deleterious effect and may be involved in the eosinophilic inflammation that characterises asthma. Experimental evidence suggests that NO may play a role in non-specific defence mechanisms against pathogens, and may be involved in the signalling between macrophages and T cells.[101] CD4+ T helper (Th) cells are important in host defence and have been implicated in chronic inflammatory diseases. Two types of Th cell are differentiated by the pattern of cytokines secreted on activation. Th1 cells release IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-10.[102 103] These patterns of cytokine production largely determine the effector functions of the two subsets of T cells.[104] Th1 cells produce IFN-γ which activates

macrophages to produce NO and kill pathogens.[105] Inhibition of NO production by analogues of L-arginine results in increased susceptibility to parasitic infections such as those produced by *Leishmania*, *Mycobacteria*, and *Plasmodium*.[106–108] IL-4 secreted by Th2 cells is of critical importance for IgE production, and is also involved in the expression of vascular cell adhesion molecule 1 (VCAM-1) which is required for the selective adhesion of eosinophils. The balance between Th1 and Th2 cells determines the outcome of many important diseases. Using cloned murine T cell lines, evidence is provided that Th1—but not Th2—cells can be activated by specific antigens to produce large amounts of NO. Furthermore, NO can inhibit the secretion of IL-2 and IFN-γ by Th1 cells but has no effect on IL-4 production by Th2 cells. Thus, NO seems to exert a self-regulatory effect on Th1 cells which are implicated in immunopathology.[109] NO derived from airway epithelial cells, macrophages, and Th1 cells plays an important role in amplifying and perpetuating the Th2 cell mediated inflammatory response, both in allergic and non-allergic asthma. iNOS may be induced in epithelial cells by exposure to pro-inflammatory cytokines such as TNF-α and IL-1β secreted by macrophages, and IFN-γ secreted by Th1 cells. It is possible that viral infections may also induce iNOS in airway epithelial cells, augmenting the secretion of NO during asthma exacerbations. The large amounts of NO generated in the airway epithelium result in suppression of Th1 cells and a concomitant reduction in the level of IFN-γ, leading to proliferation of Th2 cells. Using an allergic animal model it has been shown that the manifestations of allergic airway disease, including infiltration of inflammatory cells (eosinophils), microvascular leakage and airway occlusion, are markedly less severe in the NOS2 mutants than in wild-type animals.[110] Interestingly, the suppression of allergic inflammation was accompanied by marked increases in T cell production of IFN-γ but not by reduction in the secretion of either IL-4 or IL-5. The markedly enhanced production of IFN-γ in $NOS2^{-/-}$ mice was apparently responsible for the suppression of both eosinophils and disease, as in vivo depletion of this factor restored allergic pathology in these animals.[110] Thus, NOS2 promotes allergic inflammation in airways via downregulation of IFN-γ activity; this suggests that inhibitors of this molecule may represent a worthwhile therapeutic strategy for allergic diseases including asthma.

Recent studies have also shown that NO inhibits macrophage derived IL-12 release which is a major inducer of Th1 cells, preventing the excessive amplification of Th1 cells,[111] and also that NO generating agents increase the secretion of IL-4 in Th2 clones,[112] suggesting that, despite the complex feedback network regulating NO production, the enhanced IL-4 expression would lead to the expansion of Th2 cells once NO is generated.

NO and airway smooth muscle proliferation

In vitro studies have recently shown that exogenous administration of NO by NO donors results in reduced DNA synthesis and proliferation of airway smooth muscle cells mediated through both cGMP independent and cGMP dependent pathways.[113 114] These newly discovered antiproliferative effects of NO on airway smooth muscle may become an important feature in future strategies to prevent airway remodelling in patients with chronic asthma or COPD.

EXHALED NO AS A NON-INVASIVE BIOMARKER

NO has been detected in the exhaled breath of animals and humans by chemiluminescence.[115] The specificity of exhaled NO measurements by chemiluminescence has been confirmed using gas chromatography mass spectroscopy.[116] These reports noted the physiological capability of the lungs to release NO and have resulted in many studies of the levels of exhaled NO in disease. Increased levels of exhaled NO have been described in patients with asthma compared with healthy controls[117 118] and, in particular, the presence of atopy seems to be associated with increased levels of exhaled NO.[119 120] The increased levels of exhaled NO in asthma originate predominantly in the lower airway[121 122] and are related to airway eosinophilic inflammation[123] and to increased expression of corticosteroid sensitive iNOS.[124] Furthermore, levels of exhaled NO may reflect exacerbations[125 126] and disease severity.[127] Other disorders associated with increased exhaled NO levels include bronchiectasis,[128] rhinitis,[129] and acute lung allograft rejection.[130] In contrast, low levels of exhaled NO have been reported in patients with cystic fibrosis,[131 132] PiZZ phenotype related α_1-antitrypsin deficiency,[133] and pulmonary hypertension.[134] Data on exhaled NO in COPD are inconsistent, with both increased[135] and decreased[136] levels having been reported compared with control values. Others have reported that expired NO levels in COPD do not differ from those measured in controls.[137] The use of patient groups with differing disease severity and the inclusion or exclusion of current smokers in the analyses may explain the differences in exhaled NO levels in patients with COPD.[138] Specifically, disease severity may be an important factor since increased exhaled NO levels have been reported in patients admitted to hospital with an exacerbation of COPD, whereas NO levels were found not to differ from control values months after discharge of these patients.[139]

Several studies have been performed to assess the relationship between levels of exhaled NO and lung function parameters and other markers of airway inflammation. Exhaled NO in patients with asthma is correlated with sputum eosinophils[140 141] and airway hyperresponsiveness to methacholine,[123 142] as well as peak flow variability.[143] Furthermore, exhaled NO levels are associated with eosinophilic airway inflammation as determined in bronchoalveolar lavage fluid,[143] blood,[144] and urine[141] in patients with asthma of varying disease severity. In other studies no significant relationship has been seen between exhaled NO levels and eosinophils in bronchial biopsy specimens,[145] indicating that increased exhaled NO levels reflect some, but not all, aspects of airway inflammation. Further work is needed to determine how it relates to other markers of airway inflammation.

Exhaled NO has been used to monitor asthma exacerbations, both spontaneous[125] and those induced by steroid reduction,[146 147] and the effect of anti-inflammatory treatment in asthma.[127] It can be postulated that asthma treatment with corticosteroids results in a reduction in expired NO levels due to both the reducing effects of steroids on the underlying airways inflammation in asthma and inhibitory effects on iNOS expression itself. Oral and inhaled corticosteroids have been shown to result in a rapid (after 6 h following a single corticosteroid treatment)[148] and dose dependent reduction.[127 143 146 149 150] Since low doses of inhaled steroids (400 μg budesonide) are sufficient to reduce increased exhaled NO levels to normal values in patients with intermittent or mild persistent asthma,[149] the question arises whether these low NO levels reflect optimal control of the underlying airways inflammation or are just switching off of iNOS expression. In patients with more severe persistent asthma, the airway inflammatory processes may overcome this sensitivity of NO to steroids, resulting in increased levels of exhaled NO even during treatment with high doses of oral or inhaled corticosteroids.[146] Treatment with leukotriene antagonists has also been shown to result in a reduction in exhaled NO levels in asthma,[151 152] although this effect is not as impressive as that obtained with steroids.[152 153] However, treatment with a leukotriene receptor antagonist prevented the increase in exhaled NO levels during steroid withdrawal in patients with asthma.[154] At present there is no clear evidence of direct actions of leukotrienes on NOS expression or activity. This would indicate that, during antileukotriene

treatment, changes in exhaled NO levels more truly reflect changes in the inflammatory state of the airways.

CONCLUSIONS

Nitric oxide has a number of roles in the airways, varying from an endogenous modulator of airway function to a pro-inflammatory and immunomodulatory mediator in pathophysiological conditions. Its actions may be determined by the concentrations generated under specific circumstances, and the location and timing of synthesis. Several reports have pointed out the pathobiological importance of NO in specific airway diseases such as bronchial asthma. The bronchoprotective effects of NO in asthma include airway smooth muscle relaxation and inhibition of smooth muscle proliferation. A deficiency of local NO, probably of cNOS derived NO,[155] may be responsible for the increased AHR in asthma. On the other hand, asthma is associated with locally increased production of NO generated from overexpressed iNOS which has detrimental effects within the airways. The non-invasive measurement of NO in exhaled air seems accurately to reflect inflammation in the airways and may be of value in monitoring airway diseases such as asthma.

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