

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 humans⁷ 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 (H₄B), 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 Ca²⁺- 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)-1 β .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 \leq nM concentrations) and the lability of NO in the presence of oxygen. NO is currently measured by spectroscopic and electro-analytical 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 nitrosyl-haemoglobin (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 NK₁ 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 Ca²⁺ free medium, which suggests that a Ca²⁺ 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- κ B activation of iNOS gene⁷³ 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 $\text{TNF-}\alpha$ 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 $\text{TNF-}\alpha$ and $\text{IL-1}\beta$.⁹⁸ The stimulatory effect of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ 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 $\text{CD4+ T helper (Th)}$ 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 $\text{IFN-}\gamma$, 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 $\text{IFN-}\gamma$ 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-1 β 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 inflammation¹²³ 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 increased¹³⁵ and decreased¹³⁶ 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 urine¹⁴¹ 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 spontaneous¹²⁵ 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 μ g 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.

REFERENCES

- Culotta E, Koshland DE Jr. NO news is good news. *Science* 1992;**258**:1862–5.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;**327**:524–6.
- Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993;**329**:2002–12.
- Barnes PJ, Belvisi MG. Nitric oxide and lung disease. *Thorax* 1993;**48**:1034–43.
- Jorens PG, Vermeire PA, Herman AG. L-arginine-dependent nitric oxide synthase: a new metabolic pathway in the lung and airways. *Eur Respir J* 1993;**6**:258–66.
- Gaston B, Drazen JM, Loscalzo J, et al. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 1994;**149**:538–51.
- Gustafsson LE, Leone AM, Persson MG, et al. Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. *Biochem Biophys Res Commun* 1991;**18**:852–7.
- Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J* 1993;**6**:1368–70.
- Hobbs AJ, Ignarro LJ. The nitric oxide-cyclic GMP signal transduction system. In: Zapol WM, Bloch KD, eds. *Nitric oxide and the lung*. New York: Marcel Dekker, 1997: 1–57.
- Lamas S, Michel T. Molecular biological features of nitric oxide synthase isoforms. In: Zapol WM, Bloch KD, eds. *Nitric oxide and the lung*. New York: Marcel Dekker, 1997: 59–73.
- Marletta MA. Nitric oxide synthase structure and mechanism. *J Biol Chem* 1993;**268**:12231–4.
- Abu-Soud HM, Stuehr DS. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc Natl Acad Sci USA* 1993;**90**:10769–72.
- Förstermann U, Schmidt HH, Pollock JS, et al. Isoforms of nitric oxide synthase: characterization and purification from different cell types. *Biochem Pharmacol* 1991;**42**:1849–57.
- Morris SM, Billiar TR. New insights into the regulation of inducible nitric oxide synthase. *Am J Physiol* 1994;**266**:E829–39.
- Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 1997;**100**:2146–52.
- Ignarro LJ, Kadowitz PJ. The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annu Rev Pharmacol Toxicol* 1985;**25**:171–91.
- Hibbs JB, Taintor RR, Vavrin Z, et al. Nitric oxide: a cytotoxic activated macrophages effector molecule. *Biochem Biophys Res Commun* 1988;**157**:87–94.
- Karupiah G, Xie Q, Buller RML, et al. Inhibition of viral replication by interferon-gamma induced nitric oxide synthase. *Science* 1993;**261**:1445–8.
- Denis M. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. *Cell Immunol* 1991;**131**:150–7.
- Kwon NS, Stuehr DJ, Nathan CF. Inhibition of tumor cell ribonucleotide reductase by macrophage derived nitric oxide. *J Exp Med* 1991;**174**:761–8.
- Wink DA, Kasprzak KS, Maragos CM, et al. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 1991;**254**:1001–3.
- Radi R, Beckman JS, Bush KM, et al. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991;**288**:481–7.
- Ignarro LJ, Fukuto JM, Griscavage JM, et al. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate, comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci USA* 1993;**90**:8103–7.
- Stamler JS, Simon DI, Osborne JA, et al. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci USA* 1992;**89**:444–8.
- Zafiriou OC, McFarland M. Determination of trace levels of nitric oxide in aqueous solution. *Anal Chem* 1980;**52**:1662–7.
- Ignarro LJ, Buga GM, Wood KS, et al. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987;**84**:9265–9.
- Henry Y, Ducrocq C, Drapier J-C, et al. Nitric oxide, a biological effector: Electron paramagnetic resonance detection of nitroso-iron-protein complexes in whole cells. *Eur Biophys J* 1991;**20**:1–15.
- Archer S. Measurement of nitric oxide in biological models. *FASEB J* 1993;**7**:349–60.
- Malinski T, Czuchajowski L. Nitric oxide measurement by electrochemical methods. In: Feelish M, Stamler JS, eds. *Methods in nitric oxide research*. London: John Wiley & Sons, 1996: 319–39.
- Gruetter CA, Childers CC, Bosserman MK, et al. Comparison of relaxation induced by glyceryl trinitrate, isosorbide dinitrate and sodium nitroprusside in bovine airways. *Am Rev Respir Dis* 1989;**139**:1192–7.
- Dupuy PM, Shore SA, Drazen JM, et al. Bronchodilator action of inhaled nitric oxide in guinea pigs. *J Clin Invest* 1992;**90**:421–8.
- Högman M, Frostell C, Arnberg H, et al. Inhalation of nitric oxide modulates methacholine-induced bronchoconstriction in the rabbit. *Eur Respir J* 1993;**6**:177–80.
- Högman M, Frostell C, Hedenström H, et al. Inhalation of nitric oxide modulates adult human bronchial tone. *Am Rev Respir Dis* 1993;**148**:1474–8.
- Jansen A, Drazen J, Osborne JA, et al. The relaxant properties in guinea pig airways of S-nitrosothiols. *J Pharmacol Exp Ther* 1992;**261**:154–60.
- Gaston B, Reilly J, Drazen JM, et al. Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc Natl Acad Sci USA* 1993;**90**:10957–61.
- Gaston B, Drazen JM, Jansen A, et al. Relaxation of human bronchial smooth muscle by S-nitrosothiols in vitro. *J Pharmacol Exp Ther* 1993;**268**:978–84.
- Gaston B, Sears S, Woods J, et al. Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure. *Lancet* 1998;**351**:1317–9.
- Stretton D. Nonadrenergic, noncholinergic neural control of the airways. *Clin Exp Pharmacol Pathol* 1991;**18**:675–84.
- Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 1990;**347**:768–70.
- Fischer A, Mundel P, Mayer B, et al. Nitric oxide synthase in guinea pig lower airway innervation. *Neurosci Lett* 1993;**149**:157–60.
- Fischer A, Mayer B, Kummer W. Nitric oxide synthase in vagal sensory and sympathetic neurons innervating the guinea-pig trachea. *J Auton Nerv Syst* 1996;**56**:157–60.
- Ward JK, Barnes PJ, Springall DR, et al. *Am J Respir Cell Mol Biol* 1995;**13**:175–84.
- Li CG, Rand MJ. Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. *Br J Pharmacol* 1991;**102**:91–4.
- Belvisi MG, Stretton CD, Yacoub M, et al. Nitric oxide is the endogenous neurotransmitter of bronchodilator nerves in humans. *Eur J Pharmacol* 1992;**210**:221–2.
- Ellis JL, Udem BJ. Inhibition by L-N^G-nitro-L-arginine of nonadrenergic-noncholinergic-mediated relaxations of human isolated central and peripheral airways. *Am Rev Respir Dis* 1992;**146**:1543–7.
- Belvisi MG, Stretton CD, Barnes PJ. Nitric oxide modulates cholinergic neurotransmission in guinea-pig tracheal smooth muscle. *Eur J Pharmacol* 1991;**198**:219–22.
- Ward JK, Belvisi MG, Fox AJ, et al. Modulation of cholinergic neural bronchoconstriction by endogenous nitric oxide and vasoactive intestinal peptide in human airways in vitro. *J Clin Invest* 1993;**92**:736–42.
- Lei Y-H, Barnes PJ, Rogers DF. Regulation of NANC neural bronchoconstriction in vivo in the guinea-pig: involvement of nitric oxide, vasoactive intestinal peptide and soluble guanylyl cyclase. *Br J Pharmacol* 1993;**108**:228–35.
- Miura M, Yamauchi H, Ichinose M, et al. Impairment of neural nitric oxide-mediated relaxation after antigen exposure in guinea pig airways in vitro. *Am J Respir Crit Care Med* 1997;**156**:217–22.
- Nijkamp FP, van der Linde HJ, Folkerts G. Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro. *Am Rev Respir Dis* 1993;**148**:727–34.
- Ricciardolo FLM, Nadel JA, Yoshihara S, et al. Evidence for reduction of bradykinin-induced bronchoconstriction in guinea pigs by release of nitric oxide. *Br J Pharmacol* 1994;**113**:1147–52.
- Ricciardolo FLM, Rado V, Fabbri LM, et al. Bronchoconstriction induced by citric acid inhalation: role of tachykinins, bradykinin and nitric oxide. *Am J Respir Crit Care Med* 1999;**159**:557–62.
- Ricciardolo FLM, Trevisani M, Geppetti P, et al. NKA-induced bronchoconstriction in vivo is reduced by nitric oxide release via septide-insensitive NK1 receptor activation. *Br J Pharmacol* 2000;**129**:915–20.
- Ricciardolo FLM, Steinhoff A, Amadesi S, et al. Presence and bronchomotor activity of protease activated receptor-2 (PAR-2) in guinea pig airways. *Am J Respir Crit Care Med* 2000;**161**:1672–80.
- Persson M, Friberg SG, Hedqvist P, et al. Endogenous nitric oxide counteracts antigen-induced bronchoconstriction. *Eur J Pharmacol* 1993;**249**:R7–8.

- 56 **Persson MG**, Friberg SG, Gustafsson LE, *et al.* The promotion of patent airways and inhibition of antigen-induced bronchial obstruction by endogenous nitric oxide. *Br J Pharmacol* 1995;**116**:2957–62.
- 57 **Mehta S**, Lilly CM, Röllenhagen JE, *et al.* Acute and chronic effects of allergic airway inflammation on pulmonary nitric oxide production. *Am J Physiol* 1997;**272**:L124–31.
- 58 **Figini M**, Ricciardolo FLM, Javdan P, *et al.* Evidence that epithelium-derived relaxing factor released by bradykinin in guinea-pig trachea is nitric oxide. *Am J Respir Crit Care Med* 1996;**153**:918–23.
- 59 **Filep JG**, Battistini B, Sirois P. Induction by endothelin-1 of epithelium-dependent relaxation of guinea-pig trachea in vitro: role for nitric oxide. *Br J Pharmacol* 1993;**109**:637–44.
- 60 **Emanuelli C**, Ricciardolo FLM, Vergnani L, *et al.* Characterization of the endothelin receptor subtypes mediating relaxant NO release from guinea pig trachea. *Br J Pharmacol* 1998;**125**:963–8.
- 61 **Figini M**, Emanuelli C, Bertrand C, *et al.* Evidence that tachykinins relax the guinea-pig trachea via nitric oxide release and by stimulation of a septide-insensitive NK1 receptor. *Br J Pharmacol* 1996;**117**:1270–6.
- 62 **Ali S**, Metzger WJ, Olanrewaju HA, *et al.* Adenosine receptor-mediated relaxation of rabbit airway smooth muscle: a role for nitric oxide. *Am J Physiol* 1997;**273**:L581–7.
- 63 **Ninomiya H**, Uchida Y, Endo T, *et al.* The effects of calcitonin gene-related peptide on tracheal smooth muscle of guinea-pigs in vitro. *Br J Pharmacol* 1996;**119**:1341–6.
- 64 **Ricciardolo FLM**, Vergnani L, Wiegand S, *et al.* Detection of bradykinin-induced nitric oxide release in the airways of guinea pigs by porphyrinic microsensor. *Am J Respir Cell Mol Biol* 2000;**22**:97–104.
- 65 **Cockcroft DW**, Ruffin RW, Dolovich J, *et al.* Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 1977;**7**:503–13.
- 66 **Folkerts G**, Busse WW, Nijkamp FP, *et al.* Virus-induced airway hyperresponsiveness and asthma. *Am J Respir Crit Care Med* 1998;**157**:1708–20.
- 67 **de Boer J**, Meurs H, Coers W, *et al.* Deficiency of nitric oxide in allergen-induced airway hyperreactivity to contractile agonists after the early asthmatic reaction: an ex vivo study. *Br J Pharmacol* 1996;**119**:1109–16.
- 68 **Schiling M**, Zuidhof AB, Bonouvrie MAA, *et al.* Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs. *Br J Pharmacol* 1998;**123**:1450–6.
- 69 **Mehta S**, Drazen JM, Lilly CM, Endogenous nitric oxide and allergic bronchial hyperresponsiveness in guinea pigs. *Am J Physiol* 1997;**273**:L656–62.
- 70 **Folkerts G**, van der Linde, Nijkamp FP. Virus-induced airway hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. *J Clin Invest* 1995;**94**:26–30.
- 71 **Schuling M**, Meurs H, Zuidhof AB, *et al.* Dual action of iNOS-derived nitric oxide in allergen-induced airway hyperreactivity in conscious, unrestrained guinea pigs. *Am J Respir Crit Care Med* 1998;**158**:1442–9.
- 72 **Toward TJ**, Broadley KJ. Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation. *Br J Pharmacol* 2000;**131**:271–81.
- 73 **Liu S**, Adcock IM, Old RW, *et al.* Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. *Biochem Biophys Res Commun* 1993;**196**:1208–13.
- 74 **Meurs H**, Schuurman, Duyvendak M, *et al.* Deficiency of nitric oxide in polyclonal-induced airway hyperreactivity. *Br J Pharmacol* 1999;**126**:559–62.
- 75 **Ricciardolo FLM**, Geppetti P, Mistretta A, *et al.* Randomized double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. *Lancet* 1996;**348**:374–7.
- 76 **Ricciardolo FLM**, Di Maria GU, Mistretta A, *et al.* Impairment of bronchoprotection by nitric oxide in severe asthma. *Lancet* 1997;**350**:1297–8.
- 77 **Black PN**, Brodie SM. Nitric oxide and response to inhaled bradykinin in severe asthma. *Lancet* 1998;**351**:449–50.
- 78 **Taylor DA**, McGrath JL, Orr LM, *et al.* Effect of endogenous nitric oxide inhibition on airway responsiveness to histamine and adenosine-5'-monophosphate in asthma. *Thorax* 1998;**53**:483–9.
- 79 **Taylor DA**, McGrath JL, O'Connor BJ, *et al.* Allergen-induced early and late asthmatic responses are not affected by inhibition of endogenous nitric oxide. *Am J Respir Crit Care Med* 1998;**158**:99–106.
- 80 **Lee YC**, Cheon KT, Lee HB, *et al.* Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma. *Allergy* 2000;**55**:959–63.
- 81 **Ignarro LJ**. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ Res* 1989;**65**:1–21.
- 82 **Persson MG**, Gustafsson LE, Wiklund NP, *et al.* Endogenous nitric oxide as a probable modulator of pulmonary circulation and hypoxic pressor response in vivo. *Acta Physiol Scand* 1990;**140**:449–57.
- 83 **Adnot S**, Raffestin B, Eddamibi S, *et al.* Loss of endothelium-dependent relaxant activity in the pulmonary circulation of rats exposed to chronic hypoxia. *J Clin Invest* 1991;**87**:155–62.
- 84 **Giaid A**, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1995;**333**:214–21.
- 85 **Dinh-Xuan AT**, Higenbottam TW, Clelland CA, *et al.* Impairment of endothelium-dependent pulmonary artery relaxation in chronic obstructive lung disease. *N Engl J Med* 1991;**324**:1539–47.
- 86 **Cremona G**, Dinh-Xuan AT, Higenbottam T. Endothelium-derived relaxing factor and the pulmonary circulation. *Lung* 1991;**169**:185–202.
- 87 **Ozaki M**, Kawashima S, Yamashita T, *et al.* Reduced hypoxic pulmonary vascular remodeling by nitric oxide from the endothelium. *Hypertension* 2001;**37**:322–7.
- 88 **Alving K**, Fornhem C, Lundberg JM. Pulmonary effects of endogenous and exogenous nitric oxide in the pig: relation to cigarette smoke inhalation. *Br J Pharmacol* 1993;**110**:739–46.
- 89 **Charan NB**, Johnson S, Lakshminarayan S, *et al.* Nitric oxide and β -adrenergic agonist-induced bronchial arterial vasodilation. *J Appl Physiol* 1997;**82**:686–92.
- 90 **Sasaki F**, Parè P, Ernest D, *et al.* Endogenous nitric oxide influences acetylcholine-induced bronchovascular dilation in sheep. *J Appl Physiol* 1995;**78**:539–45.
- 91 **Baile EM**, McKay K, Wang L, *et al.* NO does not mediate inhibitory neural responses in sheep airway and bronchial vascular smooth muscle. *J Appl Physiol* 1998;**84**:809–14.
- 92 **Kubes P**. Nitric oxide and microvascular permeability: a continuing dilemma. *Eur Respir J* 1997;**10**:4–5.
- 93 **Kageyama N**, Miura M, Ichinose M, *et al.* Role of endogenous nitric oxide in airway microvascular leakage induced by inflammatory mediators. *Eur Respir J* 1997;**10**:13–9.
- 94 **Reid L**. Measurement of the bronchial mucous gland layer: a yardstick in chronic bronchitis. *Thorax* 1968;**15**:132–41.
- 95 **Nagaki M**, Shimura MN, Irokawa T, *et al.* Nitric oxide regulation of glycoconjugate secretion from feline and human airways in vitro. *Respir Physiol* 1995;**102**:89–95.
- 96 **Adler KB**, Fischer BM, Li H, *et al.* Hypersecretion of mucin in response to inflammatory mediators by guinea pig tracheal epithelial cells in vitro is blocked by inhibition of nitric oxide synthase. *Am J Respir Cell Mol Biol* 1995;**13**:526–30.
- 97 **Jain B**, Rubinstein I, Robbins RA, *et al.* Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem Biophys Res Commun* 1993;**191**:83–8.
- 98 **Jain B**, Rubinstein I, Robbins RA, *et al.* TNF-alpha and IL-1 beta upregulate nitric oxide-dependent ciliary motility in bovine airway epithelium. *Am J Physiol* 1995;**268**:L911–7.
- 99 **Duszyk M**, Radomski MW. The role of nitric oxide in the regulation of ion channels in airway epithelium: implications for diseases of the lung. *Free Radic Res* 2000;**33**:449–59.
- 100 **Duszyk M**. Regulation of anion secretion by nitric oxide in human airway epithelial cells. *Am J Physiol* 2001;**281**:L450–7.
- 101 **Barnes PJ**, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995;**16**:128–30.
- 102 **Mosmann TR**, Coffman RI. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;**7**:145–73.
- 103 **O'Garra A**, Arai N. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell Biol* 2000;**10**:542–50.
- 104 **Romagnani S**. Human TH1 and TH2 subsets: doubt no more. *Immunol Today* 1991;**12**:256–7.
- 105 **Mills CD**, Kincaid K, Alt JM, *et al.* M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 2000;**164**:6166–73.
- 106 **Green SJ**, Meltzer MS, Hibbs JB Jr, *et al.* Activated macrophages destroy intracellular Leishmania major amastigotes by an L-arginine-dependent killing mechanism. *J Immunol* 1990;**144**:278–83.
- 107 **Chan J**, Xing Y, Magliozzi S, *et al.* Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* 1992;**175**:1111–22.
- 108 **Taylor-Robinson AW**, Phillips RS, Severn A, *et al.* The role of TH1 and TH2 cells in a rodent malaria infection. *Science* 1993;**260**:1931–4.
- 109 **Taylor-Robinson AW**, Liew FY, Severn A, *et al.* Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *Eur J Immunol* 1994;**24**:980–4.
- 110 **Xiong Y**, Karupiah G, Hogan S, *et al.* Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. *J Immunol* 1999;**162**:445–52.
- 111 **Huang FP**, Niedbala W, Wei XQ, *et al.* Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *Eur J Immunol* 1998;**28**:4062–70.
- 112 **Chang RH**, Feng MH, Liu WH, *et al.* Nitric oxide increased interleukin-4 expression in T lymphocytes. *Immunology* 1997;**90**:364–9.
- 113 **Hamad AM**, Johnson SR, Knox AJ. Antiproliferative effects of NO and ANP in cultured human airway smooth muscle. *Am J Physiol* 1999;**277**:L910–8.
- 114 **Patel HJ**, Belvisi MG, Donnelly LE, *et al.* Constitutive expressions of type I NOS in human airway smooth muscle cells: evidence for an antiproliferative role. *FASEB J* 1999;**13**:1810–6.
- 115 **Gustafsson LE**, Leone AM, Persson MG, *et al.* Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. *Biochem Biophys Res Commun* 1991;**181**:852–7.
- 116 **Leone AM**, Gustafsson LE, Francis PL, *et al.* Nitric oxide is present in exhaled breath in humans: direct GC-MS confirmation. *Biochem Biophys Res Commun* 1994;**201**:883–7.
- 117 **Alving K**, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J* 1993;**6**:1368–70.
- 118 **Kharitonov SA**, Yates D, Robbins RA, *et al.* Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994;**343**:133–5.
- 119 **Gratziou C**, Lignos M, Dassiou M, *et al.* Influence of atopy on exhaled nitric oxide in patients with stable asthma and rhinitis. *Eur Respir J* 1999;**14**:897–901.
- 120 **Ludviksdottir D**, Janson C, Hogman M, *et al.* Exhaled nitric oxide and its relationship to airway responsiveness and atopy in asthma. *BHR-Study Group. Respir Med* 1999;**93**:552–6.

- 121 **Kharitonov SA**, Chung FK, Evans DJ, *et al.* The elevated level of exhaled nitric oxide in asthmatic patients is mainly derived from the lower respiratory tract. *Am J Respir Crit Care Med* 1996;**153**:1773–80.
- 122 **Massaro AF**, Mehta S, Lilly CM, *et al.* Elevated nitric oxide concentrations in isolated lower airway gas of asthmatic subjects. *Am J Respir Crit Care Med* 1996;**153**:1510–4.
- 123 **Jatakanon A**, Lim S, Kharitonov SA, *et al.* Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. *Thorax* 1998;**53**:91–5.
- 124 **Saleh D**, Ernst P, Lim S, *et al.* Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J* 1998;**12**:929–37.
- 125 **Massaro AF**, Gaston B, Kita D, *et al.* Expired nitric oxide levels during treatment of acute asthma. *Am J Respir Crit Care Med* 1995;**152**:800–3.
- 126 **Kharitonov SA**, O'Connor BJ, Evans DJ, *et al.* Allergen-induced late asthmatic reactions are associated with elevation of exhaled nitric oxide. *Am J Respir Crit Care Med* 1995;**151**:1894–9.
- 127 **Kharitonov SA**, Yates DH, Barnes PJ. Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. *Am J Respir Crit Care Med* 1996;**153**:454–7.
- 128 **Kharitonov SA**, Wells AU, O'Connor BJ, *et al.* Elevated levels of exhaled nitric oxide in bronchiectasis. *Am J Respir Crit Care Med* 1995;**151**:1889–93.
- 129 **Henriksen AH**, Sue-Chu M, Lingaas HT, *et al.* Exhaled and nasal NO levels in allergic rhinitis: relation to sensitization, pollen season and bronchial hyperresponsiveness. *Eur Respir J* 1999;**13**:301–6.
- 130 **Silkoff PE**, Caramori M, Tremblay L, *et al.* Exhaled nitric oxide in human lung transplantation. A noninvasive marker of acute rejection. *Am J Respir Crit Care Med* 1998;**157**:1822–8.
- 131 **Dotsch J**, Demirakca S, Terbrack HG, *et al.* Airway nitric oxide in asthmatic children and patients with cystic fibrosis. *Eur Respir J* 1996;**9**:2537–40.
- 132 **Grasemann H**, Michler E, Wallot M, *et al.* Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. *Pediatr Pulmonol* 1997;**24**:173–7.
- 133 **Malerba M**, Clini E, Cremona G, *et al.* Exhaled nitric oxide in patients with PiZZ phenotype-related α_1 -anti-trypsin deficiency. *Respir Med* 2001;**95**:520–5.
- 134 **Riley MS**, Porszasz J, Miranda J, *et al.* Exhaled nitric oxide during exercise in primary pulmonary hypertension and pulmonary fibrosis. *Chest* 1997;**111**:44–50.
- 135 **Corradi M**, Majori M, Cacciani GC, *et al.* Increased exhaled nitric oxide in patients with stable chronic obstructive pulmonary disease. *Thorax* 1999;**54**:572–5.
- 136 **Clini E**, Bianchi L, Pagani M, *et al.* Endogenous nitric oxide in patients with stable COPD: correlates with severity of disease. *Thorax* 1998;**53**:881–3.
- 137 **Rutgers SR**, van der Mark TW, Coers W, *et al.* Markers of nitric oxide metabolism in sputum and exhaled air are not increased in chronic obstructive pulmonary disease. *Thorax* 1999;**54**:576–80.
- 138 **Stark PJ**, de Gouw HWFM, Ricciardolo FLM, *et al.* Exhaled nitric oxide in COPD: glancing through a smoke screen. *Thorax* 1999;**54**:565–7.
- 139 **Agusti AG**, Villaverde JM, Toghres B, *et al.* Serial measurements of exhaled nitric oxide during exacerbations of chronic obstructive pulmonary disease. *Eur Respir J* 1999;**14**:523–8.
- 140 **Gibson PG**, Henry RL, Thomas P. Non invasive assessment of airway inflammation in children: induced sputum, exhaled nitric oxide, and breath condensate. *Eur Respir J* 2000;**16**:1008–15.
- 141 **Mattes J**, von Storm G, Reining U, *et al.* NO in exhaled air is correlated with markers of eosinophilic airway inflammation in corticosteroid-dependent childhood asthma. *Eur Respir J* 1999;**13**:1391–5.
- 142 **Dupont LJ**, Rochette F, Demedts MG, *et al.* Exhaled nitric oxide correlates with airway hyperresponsiveness in steroid-naive patients with mild asthma. *Am J Respir Crit Care Med* 1998;**157**:894–8.
- 143 **Lim S**, Jatakanon A, John M, *et al.* Effect of inhaled budesonide on lung function and airway inflammation. *Am J Respir Crit Care Med* 1999;**159**:22–30.
- 144 **Silvestri M**, Spallarossa D, Frangova Y, *et al.* Orally exhaled nitric oxide levels are related to the degree of blood eosinophilia in atopic children with mild-intermittent asthma. *Eur Respir J* 1999;**13**:321–6.
- 145 **Lim S**, Jatakanon A, Meah S, *et al.* Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in mild to moderately severe asthma. *Thorax* 2000;**55**:184–8.
- 146 **Kharitonov SA**, Yates DH, Chung KF, *et al.* Changes in the dose of inhaled steroid affect exhaled nitric oxide levels in asthmatic patients. *Eur Respir J* 1996;**9**:196–201.
- 147 **Jatakanon A**, Lim S, Barnes PJ. Changes in sputum eosinophils predict loss of asthma control. *Am J Respir Crit Care Med* 2000;**161**:64–72.
- 148 **Kharitonov SA**, Barnes PJ, O'Connor BJ. Reduction in exhaled nitric oxide after a single dose of nebulized budesonide in patients with asthma. *Am J Respir Crit Care Med* 1996;**153**:A799.
- 149 **Jatakanon A**, Kharitonov SA, Lim S, *et al.* Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 1999;**54**:108–14.
- 150 **Van Rensen EL**, Straathof KC, Veselic-Charvat MA, *et al.* Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* 1999;**54**:403–8.
- 151 **Bratton DL**, Lanz MJ, Miyazawa N, *et al.* Exhaled nitric oxide before and after montelukast sodium therapy in school-age children with chronic asthma: a preliminary study. *Pediatr Pulmonol* 1999;**28**:402–7.
- 152 **Bisgaard H**, Loland L, Oj JA. NO in exhaled air of asthmatic children is reduced by the leukotriene receptor antagonist montelukast. *Am J Respir Crit Care Med* 1999;**160**:1227–31.
- 153 **Stirling RG**, Kharitonov SA, Campbell D, *et al.* Increase in exhaled nitric oxide levels in patients with difficult asthma and correlation with symptoms and disease severity despite treatment with oral and inhaled corticosteroids. Asthma and Allergy Group. *Thorax* 1998;**53**:1030–4.
- 154 **Tamaoki J**, Kondo M, Sakai N, *et al.* Leukotriene antagonist prevents exacerbation of asthma during reduction of high-dose inhaled corticosteroid. The Tokyo Joshi-Idai Asthma Research Group. *Am J Respir Crit Care Med* 1997;**155**:1235–40.
- 155 **Ricciardolo FLM**, Timmers MC, Geppeti P, *et al.* Allergen-induced impairment of bronchoprotective nitric oxide synthesis in asthma. *J Allergy Clin Immunol* 2001;**108**:198–204.