Nitric Oxide in Health and Disease of the Respiratory System

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Ricciardolo, Fabio L. M., Peter J. Sterk, Benjamin Gaston, and Gert Folkerts. Nitric Oxide in Health and Disease of the Respiratory System. Physiol Rev 84: 731–765, 2004; 10.1152/physrev.00034.2003.—During the past decade a plethora of studies have unravelled the multiple roles of nitric oxide (NO) in airway physiology and pathophysiology. In the respiratory tract, NO is produced by a wide variety of cell types and is generated via oxidation of l-arginine that is catalyzed by the enzyme NO synthase (NOS). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO derived from the constitutive isoforms of NOS (nNOS and eNOS) and other NO-adduct molecules (nitrosothiols) have been shown to be modulators of bronchomotor tone. On the other hand, NO derived from iNOS seems to be a proinflammatory mediator with immunomodulatory effects. The concentration of this molecule in exhaled air is abnormal in activated states of different inflammatory airway diseases, and its monitoring is potentially a major advance in the management of, e.g., asthma. Finally, the production of NO under oxidative stress conditions secondarily generates strong oxidizing agents (reactive nitrogen species) that may modulate the development of chronic inflammatory airway diseases and/or amplify the inflammatory response. The fundamental mechanisms driving the altered NO bioactivity...
under pathological conditions still need to be fully clarified, because their regulation provides a novel target in the prevention and treatment of chronic inflammatory diseases of the airways.

I. INTRODUCTION

A. Historical View

The small, light, and simple molecule nitric oxide (NO) was once regarded only as a noxious environmental pollutant in cigarette smoke, smog (317), and the exhaust from motorcars, destroying the ozone layer and causing acid rain (68). This bad reputation of NO changed when in the 1980s several lines of research showed that NO is an essential molecule in the physiology of the human body.

Early studies demonstrated that endothelial cells are able to release a labile factor, named as endothelium-derived relaxing factor (EDRF), that diffuses to the adjacent muscle layer and relaxes it (124) at least in part stimulating the formation of cGMP (359). Similarly, biochemical experiments showed that nitroglycerin elicits blood vessel relaxation after its conversion to NO with the subsequent formation of cGMP (299). Finally, in 1987, the proof that NO was similar to EDRF (190, 331) was provided. Subsequently, the importance of NO and other nitrogen oxides in the regulation of various body functions, including platelet aggregation (357) and neurotransmission (40), emerged. Eventually, this set of observations was honored by the Nobel prize in 1998.

Shortly after the publication of landmark papers proposing EDRF to be NO, several investigators made observations suggesting that nitrogen oxides are relevant to respiratory biology. First, Pepke-Zaba et al. (339) initiated a successful trial using inhaled NO (ppm concentrations) as a selective pulmonary vasodilator. Simultaneously, Gustafsson et al. (156) measured endogenous NO (ppb concentrations) in the exhaled air of humans and other mammals. Working independently, Gustafsson’s group (345) and three other groups (12, 130, 236) found that NO concentrations were higher than normal in patients with asthma, but low in patients with cystic fibrosis; there was great excitement when these parallel findings were reported at the Biology of Nitric Oxide meeting in Cologne in 1992.

The increased NO levels in exhaled air of asthmatic patients might be explained by an overexpression of the enzyme that synthesizes NO (162, 242). NO can be produced by a number of cells in the airways such as endothelial cells and inflammatory cells. However, these data regarding endogenous NO in the lung represented a series of paradoxes. For example, how could the alveolar space contain NO if it was thought to “dump” out NO by virtue of hemoglobin reactivity? Or more importantly, why are the concentrations measured in expired air three log orders lower than those used to decrease pulmonary vascular resistance? A tremendous amount of research has subsequently been devoted to addressing the troubling paradoxes of pulmonary NO biology; however, many questions remained unanswered. As an example, Beall et al. (30) have recently suggested that concentrations of NO as low as 200 ppb may be relevant to subtle regulation of oxygen uptake in the lungs, but no role has been directly demonstrated for NO gas itself at physiological concentrations. In this regard, it has been argued from the time of the first studies in endogenous nitrogen oxide biology that NO itself may not be the only, or indeed the most important, product of NO synthase (NOS) activation relevant to respiratory physiology (126, 307).

In addition, NO acts also as a neurotransmitter of the inhibitory nonadrenergic noncholinergic (NANC) nerves. In human central and peripheral airways in vitro, NO appears to account for the bronchodilator NANC response (32, 92). Therefore, a physiological function of NO in the airways might be dilatation of bronchial smooth muscle. It has been known for more than half a century that nitrates induce bronchial relaxation (143). NO and NO donors relax human airway smooth muscle in vitro (151, 438), and a bronchodilatory effect of inhaled NO was demonstrated in guinea pigs and humans during methacholine-induced bronchoconstriction (85, 210).

The other way around, inhibition of NO formation increases airway responsiveness to contractile agents in animals and asthmatic patients (315, 365). Again, we face a paradox in pulmonary nitrogen oxide biology here: although the concentrations of exhaled NO are increased in patients with asthma, airway responsiveness is increased instead of suppressed. During the last few years several studies have been performed to assess the relationship between levels of exhaled NO and lung function parameters or other markers of airway inflammation.

B. Bioactive Forms of NO

NO itself has a short half-life in vivo (1–5 s) because of its reactivity with hemoglobin (223, 266, 419) and a broad spectrum of other biological compounds. It has one unpaired electron, making it a free radical that avidly reacts with other molecules such as oxygen, superoxide radicals, or transition metals. NO may be formed and/or bioactivated as nitroxyl (NO•) or nitrosonium (NO⁺). These chemical species have short half-lives in aqueous solution (<1 s) but are stabilized in biological complexes with thiols (RS− ... "NO"), nitrite (O2N− ... "NO"), and other targets and intermediates (404). Here, we will refer to NO•, NO⁺ and NO− as “NO”, unless specified otherwise. NO is an ubiquitous messenger molecule that affects vari-
ious biological functions, either at low concentrations as a signal in many physiological processes such as blood flow regulation, platelet reactivity, NANC neurotransmission, and central nervous system memory or at high concentrations as cytotoxic and cytostatic defensive mechanisms against tumors and pathogens (for references, see Ref. 298). Many studies demonstrated a significant role for these nitrogen oxides in modulating pulmonary function and in the pathogenesis of various pulmonary diseases (27, 128, 209). Moreover, NO has been detected in exhaled air of animals and humans (156), and the NO concentrations are changed in different inflammatory diseases of the airways such as asthma (12, 126, 345).

Reactions of NO ultimately lead to the nitration (addition of -NO₂), nitrosation (addition of -NO⁻), and nitrosylation (-NO) of most classes of biomolecules. One of the best known interactions of NO leading to cell signaling is the reversible covalent binding, nitrosylation, with the ferrous heme in soluble guanylyl cyclase. Another aspect of NO signaling are S-nitrosothiols (SNO) that appear to be important molecules signaling “NO” bioactivity in the lung. SNOs are products of NOS activation that are present in the airway lining fluid in micromolar concentrations, stored in specific cellular compartments to achieve bioactivity and metabolically regulated to deliver bioactivities both through transnitrosation reactions and through release of NO.

C. Regulation of NOS

NO and related compounds are produced by a wide variety of residential and inflammatory cells in the airways (129). NO itself is generated via a five-electron oxidation of terminal guanidinium nitrogen on the amino acid L-arginine (Fig. 1). The reaction is both oxygen- and
nicotinamide adenine dinucleotide phosphate (NADPH)-dependent and yields the coproduct L-citrulline in addition to nitroxyll (NO·), in a 1:1 stoichiometry (174, 392). The enzyme system responsible for producing NO, first functionally identified in 1990 by Bult et al. (46), is NOS, which exists in three distinct isoforms: 1) constitutive neuronal NOS (NOS I or nNOS); 2) inducible NOS (NOS II or iNOS); and 3) constitutive endothelial NOS (NOS III or eNOS). Protein purification and molecular cloning approaches have identified the three distinct isoforms of NOS. 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 (252). All of the three NOS isoforms are expressed in the airways (108, 162, 242, 374, 397).

Functionally, NOS exists in constitutive (cNOS) and inducible (iNOS) forms (116). cNOS is a Ca2+- and calmodulin-dependent enzyme and releases, within seconds, femtomolar or picomolar concentrations of NO upon receptor stimulation by selective agonists (Fig. 1). iNOS isoform is regulated at a pretranslational level and can be induced by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin (IL)-1β (303). iNOS releases large quantities (nM concentrations) of proinflammatory NO several hours after exposure, which may continue in a sustained manner (hours or days) (Fig. 2).

The cellular synthesis of the three archetypal enzyme isoforms appears to be dynamically regulated. Changes in NO production are correlated with similar changes in iNOS mRNA abundance, indicating that a major part of iNOS regulation occurs at a pretranslational step such as transcription or mRNA stability (303). Constitutively expressed iNOS in human airway epithelium has been shown by Asano et al. (16) and Guo et al. (154). These latter investigators noted that this unusual expression was lost when human airway epithelium was cultured (154, 155). These authors identified an autocrine mechanism of induction and maintenance of iNOS in human airway epithelial cells through the synthesis and secretion of a soluble mediator (429). Several lines of experimentation have established that transcriptional control mechanisms form an important basis for regulation of this isoform. Induction of macrophage iNOS mRNA by lipopolysaccharide (LPS) plus IFN-γ reflects increased iNOS gene transcription without changes in iNOS mRNA stability (303). In marked contrast to the effects of LPS and IFN-γ, transforming growth factor-β (TGF-β) suppresses macrophage iNOS expression via decreased iNOS mRNA stability and translational efficiency and by decreased stability of iNOS protein, but TGF-β does not alter iNOS transcription (303). Availability of molecular clones corresponding to the mouse iNOS promoter allowed, through the analysis of controlled deletions within the promoter region, the characterization of two major 5′-flanking regulatory regions, one LPS sensitive and the other IFN-γ sensitive, the latter possessing functional characteristics of an enhancer (375). The LPS-sensitive region contains a binding site for NFκB, a transcription factor that has been impli-

![FIG. 2. Overview of the signal transduction pathway leading to the increased expression of inducible nitric oxide synthase (iNOS). A variety of stimuli cause tyrosine kinase activation with subsequent activation of nuclear transcription factor NFκB via phosphorylation and degradation of inhibitory (I)κB. NFκB will accordingly be translocated to the nucleus, and this will lead to mRNA transcription of the iNOS gene. Translation of iNOS mRNA will take place with assembly of the iNOS protein as a result. L-Arginine will be metabolized to L-citrulline and nitric oxide (NO). As described in the text, NO generated by iNOS has beneficial effects (i.e., host defense) but also a number of harmful effects.](http://physrev.physiology.org/)

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lated in the activation of various genes expressed in inflammatory responses. After specific receptor (CD14) stimulation, LPS activates the mitogen-activated protein (MAP) kinase pathway with subsequent activation of NFkB through phosphorylation and degradation of IkB (Fig. 2) (272). Of note, there is evidence for feedback inhibition of this NFkB pathway by NO through two different S-nitrosylation pathways (280, 324). An upstream site contains enhancer regions with binding sites for γ-activated site (GAS) element and an IRF-1 specific response element (ISRE) that account for IFN-γ induction (270, 351). IFN-γ signaling to gene expression begins with a specific receptor interaction followed by the Janus kinase (JAK)-STAT1 pathway that involves a tyrosine phosphorylation cascade (164, 172). In fact, pretreatment with genistein, a tyrosine kinase inhibitor, prevents IFN-γ induction of iNOS expression in airway epithelial cells (153). STAT is also able to activate another transcription factor, IRF-1. Both STAT-1 and IRF-1 interact with the response elements GAS and ISRE in the iNOS promoter regions (272, 351).

Whereas transcriptional regulation of iNOS has been established for ~10 years, no expressional regulation was originally known for the other two isoforms. More recent evidence suggests, however, that the expression of nNOS and eNOS can also be regulated under various conditions. nNOS mRNA transcripts and/or protein have been detected in specific neurons of the central and peripheral nervous systems and in nonneuronal cell types such as airway epithelial cells (114). The subcellular localization of nNOS protein varies among the cell types studied. In neurons, both soluble and particulate protein is found. nNOS expression can be dynamically regulated by various physiological and pathological conditions (114). nNOS mRNA upregulation seems to represent a general response of neuronal cells to stress induced by a large array of physical, chemical, and biological agents such as heat, electrical stimulation, light exposure, and allergic substances. Enhanced nNOS expression is often associated with coinduction of transcription factors such as c-jun (455) and c-fos (422).

While iNOS has been characterized as a soluble (cytosolic) protein, eNOS is targeted to Golgi membranes and plasmalemmal caveolae (small invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin). This complex process is probably dependent on myristoylation, palmitoylation, and tyrosine phosphorylation of the enzyme as well as protein-protein interactions with caveolins (292). In endothelial cells it has been demonstrated that the association between eNOS and caveolin suppresses eNOS activity. After agonist activation the increase in intracellular Ca2⁺ concentration ([Ca2⁺]i) promotes calmodulin binding to eNOS and the dissociation of caveolin from eNOS. eNOS-calmodulin complex synthesizes NO until [Ca2⁺]i decreases and then the inhibitory eNOS-caveolin complex reforms (292). Interestingly, estrogen upregulates and activates eNOS in endothelial cells. 17β-Estradiol increases NO-dependent dilatation of rat pulmonary arteries and thoracic aorta (142), and estrogen acutely stimulates eNOS in H441 human airway epithelial cells (239). An exciting aspect of this emerging area of study is that estrogen, NO, and caveola research fields have merged to identify a novel clinical relevant molecular process (468).

D. Localization of NO in the Airways

1. eNOS (NOS III)

Soon after the identification of NO as a messenger molecule generated by endothelial cells, a calcium- and L-arginine-dependent enzyme was identified, and >95% of its activity was sequestered in the particulate fraction of the endothelial (115). Indeed, after the enzyme had been cloned and sequenced (202), and specific antisera for the endothelial isoform of NO had become available, abundant eNOS immunoreactivity was found in endothelial cells of pulmonary blood vessels. A recent review describes that eNOS is localized to endothelial caveolae by palmitoylation (395).

eNOS is constitutively expressed in human bronchial epithelium (397) and in type II human alveolar epithelial cells (337). Immunoreactivity for eNOS is also localized in the epithelium of human nasal mucosa (219). Ultrastructural studies revealed that eNOS is localized at the basal membrane of ciliary microtubules (458), where it is thought to contribute to the regulation of ciliary beat frequency (197).

2. nNOS (NOS I)

nNOS (NOS I) is localized in airway nerves of humans (78, 106, 152, 242, 271) and animals (77, 78, 152, 242, 271, 428). Substantial species differences are apparent with regard to the extent of innervation and origin of nerve fibers. In human airways, nerve fibers containing nNOS have been shown both by immunohistochemistry and NADPH-diaphorase histochemistry (106, 242, 437). These nerve fibers are present in the airway smooth muscle, where NO is the major mediator for the neural smooth muscle relaxation (32, 258). The density of these nerve fibers decreases from trachea to small bronchi (106), which is associated with a reduced neural bronchodilatation (92, 437) mediated by the inhibitory NANC (iNANC) system (446). Colocalization with vasoactive intestinal polypeptide (VIP) is frequently observed (250). In human airways, NOS-containing nerve fibers are present around submucosal glands (106), although their functional role for the regulation of glandular secretion is not
clear yet. In the lamina propria, NO has potent dilatory effects on blood vessels and on the regulation of plasma extravasation (94).

The cell bodies of these neurons innervating human airways are localized predominantly in the local parasympathetic ganglia (78, 107). Additional sources of NOS immunoreactive nerve fibers are found in vagal sensory and sympathetic ganglia (107, 249, 326). NOS immunoreactive neurons are present in vagal sensory ganglia in humans (50, 107, 249) and in rats (7). In sensory neurons, NO could act as a neuromediator both at the central ending and the periphery (382).

In the central nervous system, reports identified nNOS activity in the cytosolic fraction (114). However, a PDZ-domain has been found in the NH₂-terminal nNOS. [The abbreviation PDZ derives from the PDZ-domain has been found in the NH₂-terminal nNOS. NO could act as a neuromediator both at the central active neurons are present in vagal sensory ganglia in and sympathetic ganglia (107, 249, 326). NOS immunoreactive nerve immunoreactive nerve is also present in nonneuronal tissues like the respiratory epithelium of guinea pig and rat (94, 242) and in normal endothelial cells (267). In the pulmonary arteries and veins of rats, endothelial cells display immunoreactivity in the cytoplasm (268).

3. iNOS (NOS II)

iNOS (NOS II) has been identified as a separate, calcium-independent isoform, which could be detected in brain, lung, and liver of rats after endotoxin treatment (241). In macrophages it has been revealed by cloning and sequencing that iNOS is expressed de novo at the transcriptional level (273, 456). It is now clear that this isoform is not only localized to macrophages (338), but it can be induced in many different cells (105). In the respiratory tract alone, expression of iNOS has been reported in alveolar type II epithelial cells (440), lung fibroblasts (380), airway and vascular smooth muscle cells (150, 418, 459), airway respiratory epithelial cells (2, 337, 374, 441), mast cells (139) endothelial cells (95), neutrophils (35), and chondrocytes (242). The stimuli that cause transcriptional activation of iNOS in these cells varied widely and included endogenous mediators (such as chemokines and cytokines) as well as exogenous factors such as bacterial toxins, virus infection, allergens, environmental pollutants (ozone, oxidative stress, silica), hypoxia, tumors, etc. (Fig. 2) (140, 462, 464). The expression of iNOS in the lung can be prevented by glucocorticoids (157). In respiratory epithelial cells of human lung, a “constitutive” expression of iNOS is observed at mRNA (154) and protein level (242). Under normal conditions, however, some investigators could not detect the expression of iNOS (48). It should be stressed, however, that it is difficult to induce iNOS in human cells in vitro and that there are marked differences in the promoter region of iNOS between humans and rodents. Corticosteroids inhibit rodent iNOS, whereas in humans steroids presumably reduce the inflammatory signals that lead to the induction of iNOS.

In conclusion, all three NOS isoforms are localized in the respiratory system (16) where they may cooperatively regulate airway smooth muscle tone and immunologic/inflammatory responses.

E. Arginine Uptake and Metabolism

Because l-arginine is the only physiological substrate for NOS, regulation of l-arginine availability could determine cellular rates of NO production. l-Arginine is an essential amino acid, which is supplied by the diet and actively transported into the cell. l-Arginine displays affinity for the cationic amino acid transporter in various cell types, but the correlation between l-arginine transport and its availability as a substrate for NO synthesis is not well understood (301, 453).

A high-affinity carrier resembling the cationic amino acid transport (CAT) system y⁺ is likely to be responsible for the transcellular transport of arginine (Fig. 1), with minor roles being played by systems b₀⁺⁺, B₀⁺⁺, and y⁺L (76). The physiological hallmarks of system y⁺ are the high affinity for amino acids with a positively charged side chain, its independence from the concentration of extracellular Na⁺, and the trans-stimulation of arginine transport by the other cationic amino acids l-lysine and l-ornithine. This system has been detected in many cells, among them macrophages, endothelial cells, platelets, and vascular smooth muscle cells (447). System y⁺ activity is mediated by the CAT family that is composed of four isoforms, CAT-1, CAT-2A, CAT-2B, and CAT-3 (301). NOS inhibitors based on a modification of the arginine structure (with a positive charge) are also transported by system y⁺. Moreover, arginine itself is a proteinogenic amino acid and, once incorporated into proteins, can be posttranslationally N⁶-methylated to the NOS inhibitors N⁶-monomethyl-l-arginine (L-NMMA) (exogenous) and asymmetric dimethylarginine (ADMA) (endogenous) or deaminated to form citrulline (447).

The activation of l-arginine transport is sensitive to cycloheximide, demonstrating that de novo protein synthesis is essential for enhanced transporter activity. l-Arginine transport in tissues and many different cell types, such as vascular smooth muscle cells and macrophages, can be stimulated by LPS, but is hardly affected by TNF-α, IL-1β, or IFN-γ (for an overview, see Ref. 301). These findings suggest that induction of iNOS and l-arginine transporting activity are dependent on the stimulus used, with an adequate combination of cytokines and/or LPS being responsible for full activation of one or both pathways (Fig. 1). Dexamethasone selectively inhib-
its the production of NO produced by iNOS whilst having no effect on transport, indicating that the gene for the L-arginine transporter is not sensitive to regulation by glucocorticoids (449). L-Arginine is abundant with a normal dietary intake, but its availability is low owing to extensive protein binding. Oral administration of L-arginine to humans is associated with an increased concentration of NO in exhaled air and was associated with an increase in the concentration of L-arginine and nitrate in plasma (230, 388). These results suggest that an increase in the amount of substrate for NO can increase the formation of endogenous NO.

Arginine can be metabolized by two groups of enzymes. As mentioned above arginine can be converted by NOS to citrulline but can also be catabolized by arginase (Fig. 1).

Arginase exists in two isoforms, liver-type arginase I (165, 220) and nonhepatic type arginase II (36, 302, 435). Arginase I is localized in the cytosol, and arginase II is located in the mitochondrial matrix. iNOS and arginase II are coinduced in LPS-stimulated RAW 264.7 macrophages (304). Moreover, arginase I but not arginase II is coinduced with iNOS in rat peritoneal macrophages and in vivo in rat lung after LPS treatment. In mouse bone marrow-derived macrophages, NOS and arginase activities are regulated by T-helper 1 (Th1) and Th2 cytokines, respectively (297). Moreover, arginase can be induced in the lungs of rats after hyperoxia (355). Allergy is considered to be a Th2-mediated disease, and indeed, arginase activity is increased 3.5-fold in the lungs of guinea pigs after ovalbumin sensitization and challenge (290). Meurs et al. (290) hypothesized that the corresponding airway hyperresponsiveness in these animals is caused by a NO deficiency due to the increased arginase activity (290). Indeed, pretreatment of the tissues with the arginase inhibitor Nω-hydroxy-nor-arginine (nor-NOHA) suppressed the allergen-induced airway hyperresponsiveness (290). Interestingly, Nω-hydroxy-L-arginine (NOHA) is an intermediate in the biosynthesis of NO (Fig. 1) (36, 45). LPS-treated rat alveolar macrophages produce high amounts of NOHA (166, 169). The inhibition of arginase by NOHA may ensure sufficient high-output production of NO in activated macrophages, which may be important for the killing of microorganisms. On the other hand, a high production of NO is toxic for cells, and arginase I and mitochondrial arginase II prevent NO-mediated apoptosis in activated macrophages. Therefore, a delicate balance between the beneficial and harmful pathophysiological effects of NO exists in the airways, which might be regulated by arginine metabolism.

F. Molecular Action of NO

NO bioactivities are broadly classified as NO mediated/cGMP dependent and cGMP independent. Many bioactivities, such as airway smooth muscle relaxation, appear to use both. Relaxation of human airway smooth muscle by NO, released as a neurotransmitter, may be partially mediated via cGMP (438). However, airway smooth muscle relaxation to NO and other nitrogen oxides has also been shown to be a cGMP-independent process in humans and a variety of other species (127, 200, 341, 421). cGMP-independent bioactivities, ranging from neurotransmission to cell cycle regulation, appear to involve NO reactivity with alternate metal centers and transfer of an NO+ (nitrosonium) equivalent from one thiol group to another to up- or downregulate target protein function.

Chemical features of NO radical include its rapid diffusion from the point of synthesis, the ability to permeate cell membranes, the interactions with intracellular molecular sites within both generating and target cells, and its intrinsic instability, all properties that eliminate the need for extracellular NO receptors or targeted NO degradation. The best-characterized target site for NO is the iron bound in the heme component of soluble guanylyl cyclase stimulating conversion of GTP to cGMP and mediating the biological effects attributed to eNOS-derived NO (191). Subsequently, cGMP exerts most of the intracellular actions by coupling to cGMP-dependent protein kinase (PKG). It is generally accepted that cGMP triggers relaxation of smooth muscle by activating two molecular mechanisms: reduction of 

\[ \text{Ca}^{2+}\text{c}_{i} \] and reduction of the sensitivity of the contractile system to the Ca\textsuperscript{2+}. The former is due to the ability of activated PKG to phosphorylate several key target proteins with the final effect of reducing \[ \text{Ca}^{2+}\text{c}_{i} \]. In particular, PKG may stimulate Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (\( K_{\text{ca}} \)), inhibit membrane Ca\textsuperscript{2+} channel activity, activate Ca\textsuperscript{2+}-ATPase pump in the plasma membrane and in the sarcoplasmatic reticulum, and inhibit inositol trisphosphate receptor and generation (55). The mechanism of the cGMP-induced \( \text{Ca}^{2+} \) desensitization is mainly ascribed to the stimulation of myosin light-chain phosphatase activity via inhibition of RhoA-dependent pathway (391). In addition, NO mediates other actions that are independent of guanylyl cyclase and cGMP. The high level of NO released by iNOS has an effect as immune effector molecule in killing tumor cells (170), in halting viral replication (216), and in eliminating various pathogens. In fact, NO has been reported to inhibit the growth of or kill a number of fungi, parasites, and bacteria including Mycobacterium tuberculosis (73). This mechanism may involve, at least in part, inhibition of DNA synthesis by inactivation of ribonucleotide reductase and by direct deamination of DNA (235, 451). Finally, NO appears to signal through its reactivity with cysteine groups, particularly those located at consensus motifs for S-nitrosylation with primary sequence or tertiary structure of a protein (Fig. 1) (see below) (340, 405). One of the general mechanisms of antimicrobial defenses involving
NO is S-nitrosylation by NO of cysteine proteases, which are critical for virulence, or replication of many viruses, bacteria, and parasites (390).

Interaction of NO with many molecular targets also may represent a pathway for its breakdown and inactivation. The most important interaction is probably its reaction with superoxide anion (O$_2^-$) to yield peroxynitrite anion (ONOO$^-$), which is a potent cytotoxic molecule (356).

G. Regulation of SNO-Mediated Bioactivities

Pulmonary SNO bioactivities are generally those in which functional protein modification is caused by NO transfer to a cysteine thiol (Fig. 1). Specificity of this signaling is achieved by regulation of synthesis, compartmentalization, compositional balance, and catabolism. S-nitrosothiol synthesis may be regulated following NOS activation by proteins such as ceruloplasmin, hemoglobin, and albumin (145, 193, 358) and/or NOS itself (144, 392). Specific compartments of relevance are, for example, the mitochondrial intermembrane space, where S-nitrosylated caspases are sequestered before being released into the reducing environment of the cytosol and thereby activated by reductive cleavage of the SNO bond (277, 278). Compositional specificity is reflected in the requirement of S-nitrosoglutathione (GSNO) to be cleaved to S-nitrosocysteineglycine, and thereby activated for intracellular transport, by $\gamma$-glutamyltranspeptidase (GGT) (18, 261). S-nitroso-l-cysteine is highly bioactive in S-nitrosylating specific airway epithelial cell proteins, relaxing pulmonary vascular smooth muscle, and increasing neuronal signaling to increased minute ventilation response to hypoxia, in a GGT-independent fashion (261), whereas the d-isomer of S-nitrosocteine (CSNO) is completely nonfunctional in all of these bioactivities (261, 323). Note in this regard that the l- and d-isomers of CSNO release NO at the same rate. Finally, catabolic degradation by the activity of glutathione disulfide reductase (GSSG) and ammonia, regulates cellular levels of S-nitrosylated protein (264).

II. NITRIC OXIDE AND PHYSIOLOGY OF THE RESPIRATORY SYSTEM

A. NO and Lung Development

Spatial and temporal nNOS and eNOS expression patterns occur during development of the lung (218, 460). Quantitative developmental studies of mRNA and protein expression as well as immunohistochemical examination revealed that the eNOS isoform increases during fetal development of the lung (159, 173, 218). In fetal lungs of sheep, eNOS expression was evident in bronchial and proximal epithelia but was absent in terminal and respiratory bronchioles and alveolar epithelium (398). The latter data were confirmed by isoform-specific reverse transcription-polymerase chain reaction assays and NADPH diaphorase histochemistry, which excludes misinterpretation due to immunohistochemistry (64). It was speculated that the rise in fetal lung eNOS may contribute to the marked lung growth and angiogenesis that occurs during the same period of time (333). Shaul et al. (396) suggested that the increase in nNOS and eNOS in the lung early in the third trimester in the primate may enhance airway and parenchymal function in the immediate postnatal period.

B. NO and Transcriptional Regulation in the Lung

S-nitrosylation reactions appear to be of particular relevance to regulation of gene expression in the lung (Fig. 1). Several examples are provided. First, SNOs associated with hemoglobin deoxygenation (261, 335) appear to stabilize the a-subunit of hypoxia-inducible factor 1 (HIF 1) (330) through increased HIF 1 DNA binding activity, in turn increasing downstream expression of hypoxia-inducible genes such as vascular endothelial growth factor in the pulmonary vascular endothelium. Of note, this system only requires SNO formation through hemoglobin deoxygenation rather than the profoundly low oxygen tension, generally <7 mmHg and not relevant in the airway or pulmonary vasculature, required conventionally in vitro (194) to activate HIF 1. Second, physiological levels of GSNO increase DNA binding of gene regulatory protein SP1 and downstream transcription of housekeeping genes such as that for the cystic fibrosis transmembrane regulatory protein (CFTR), while supraphysiological concentrations (>10 $\mu$M) completely inhibit SP1 binding, shutting off transcription of housekeeping genes perhaps to redirect cellular resources to stress response. These observations may have relevance to the effect of high levels of nitrosylating agents in the lung, which paradoxically inhibit wild-type CFTR expression at the transcriptional level (467). Third, high levels of nitrosative stress can inhibit NFkB inactivation through direct S-nitrosylation or through S-nitrosylation of IkB kinase (280, 324). These signaling mechanisms may serve to control cytokine production under physiological conditions, while increasing cytokine production during periods of nitrosative stress.

C. NO and iNANC

Cholinergic and adrenergic systems control the bronchomotor tone together with the NANC system which
mediates contraction [excitatory NANC (eNANC)] or relaxation (iNANC) of airway smooth muscle (39, 408). Recent evidence has shown that NO is a neurotransmitter of iNANC system and that nitricergic neurotransmission is present in several organs including the airways (27). Immunostaining studies demonstrated that nNOS is localized into nerves of guinea pig and human airways (242) which supply vessels, smooth muscle, and lamina propria (108). NOS immunoreactive neurons are found in parasympathetic ganglia and also in sympathetic and sensory (more in jugular than in nodose) ganglia supplying the airways (107, 108). They are more prominent in proximal than in distal airways (437), in agreement with the distribution of iNANC functional responses (108). NO is released from peripheral nerves by nNOS and is activated by calcium entry when the nerve is depolarized (41).

NO mediates approximately one-half of the iNANC (relaxant) response in guinea pig trachea in vitro, and the neuropeptide VIP should be involved in the second half of iNANC relaxant response (258). Of note, VIP-mediated guinea pig airway smooth muscle relaxation is preceded by release of SNOs into the airways (259). The human iNANC response in central and peripheral airways is completely mediated by NO (32, 92). In addition, it has been shown in human airways that iNANC bronchodilator response evoked by electrical field stimulation is associated with a concurrent increase in cGMP content in smooth muscle cells reflecting a cGMP-dependent pathway of neurogenic NO in modulating airway caliber (438). It has also been found that NO-dependent iNANC relaxations are due to the selective activation of K_Ca channels in airway smooth muscle (213). NOS may be colocalized with VIP (250, 399), which can also stimulate NO/SNO production (259, 426). The neurons, which release NO, are probably part of the cholinergic pathway. However, stimulation of the preganglionic cervical vagus nerve in an in vitro guinea pig tracheal tube preparation did not cause NO-mediated bronchodilation, while activation of postganglionic intrinsic nerves provoked bronchodilatation, suggesting that NO-dependent NANC relaxations of the airways are mediated by postganglionic parasympathetic nerves (442). Recently, it has been shown that a NO-dependent component of noncholinergic parasympathetic nerves modulates airway smooth muscle tone at baseline, pointing out the spontaneous activity of noncholinergic nerves during tidal breathing (225). Fischer et al. (104) provided the first evidence that NOS-immunoreactive neurons intrinsic to the guinea pig esophagus project axons to the adjacent trachealis, showing that these neurons could be the postganglionic parasympathetic neurons mediating iNANC relaxation of the trachealis. Furthermore, inhibition of NOS potentiates cholinergic neural bronchoconstriction (31, 439). However, it does not change neural acetylcholine release (31, 439), suggesting that nNOS-derived NO is a functional antagonist to excitatory cholinergic pathway at the postjunctional, and not the prejunctional, level (175).

Physiological and morphological studies of iNANC nerves indicate that they represent a distinct parasympathetic pathway from the well-characterized cholinergic-parasympathetic pathways innervating the airways (52, 175). Consequently, it seems likely that interactions between these nerve pathways occur postjunctionally and are manifested through their opposing actions on airway smooth muscle. In particular, Canning et al. (51) observed that stimulation of capsaicin-sensitive visceral afferent fibers activates, upon peripheral release of tachykinins, iNANC neurons innervating guinea pig trachealis via activation of both NK_{3} and NK_{1} receptors (51). It has also been observed that endogenous NO released in association with nerve stimulation regulates the magnitude of eNANC response in guinea pig airways (254). In a recent study it has been observed that the nonadrenergic bronchodilatation induced by capsaicin is suppressed by the NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME) providing the first evidence of iNANC-derived NO modulation in airway responsiveness of cats in vivo (8).

The fact that in human airways iNANC nerves are the sole neural bronchodilator pathway leads to the hypothesis that any impairment of these nerves such as in inflammatory states has functional consequences for airway patency. Indeed, iNANC responses are significantly reduced from patients with cystic fibrosis, in which there is an intense neutrophilic inflammation of the airways, compared with iNANC responses in normal tissue (27). Furthermore, it has been noted that the circadian variations of the iNANC response may contribute to overnight bronchoconstriction in patients with nocturnal asthma (274). Neural NO-induced relaxation is impaired in guinea pig airways after allergen exposure, without affecting nNOS expression, suggesting a reduced neural NOS activity when allergic inflammation is exacerbated (296).

D. NO and Airway Smooth Muscle Relaxation

The ability of NO to relax smooth muscle has been described in multiple models and muscle types, including airway smooth muscle (55). More than half a century ago, nitrates were supposed to induce bronchial relaxations (143). In 1968 Aviado et al. (19) demonstrated that nitrates were supposed to induce bronchial relaxations (49, 224, 293, 325). Gruetter et al. (151) have shown that nitrovasodilators induce relaxation of isolated airway smooth muscle, activate guanylyl cyclase, and raise cGMP levels. In anesthetized guinea pigs, methacholine-induced bronchoconstriction is reduced by inhaled
NO in a concentration-dependent manner from 5 to 300 ppm (85). In addition, a high concentration of NO (300 ppm) causes a small degree of baseline bronchodilatation. Furthermore, in anesthetized and mechanically ventilated rabbits, 80 ppm NO added to the inspired gas prevents increased resistance in response to nebulized methacholine (177). In contrast, there is no effect on pulmonary compliance, suggesting that NO prevents the contraction of the larger airways to a greater extent than the small airways (177). Inhaled NO at a concentration of 80 ppm has no effect in normal human subjects and in chronic obstructive pulmonary disease (COPD) patients, but a small bronchodilator effect in asthmatic patients (178). NO-dependent airway relaxation is partially due to activation of $K_{c a}$ channels via guanylyl cyclase and PKG (461). Moreover, these relaxations are due to inhibition of $Ca^{2+}$ release, after stimulation of inositol trisphosphate receptors and ryanodine receptors, from sarcoplasmic reticulum of airway smooth muscle cells mediated via cGMP-dependent mechanisms (214).

Interestingly, there is increasing evidence for another mechanism, in addition to guanylyl cyclase activation, by which NO relaxes human bronchial smooth muscle (24, 127, 199, 341, 421). One of the metabolic pathways for NO also involves its reaction in the presence of thiol to form SNOs (126). SNOs are present in the airways of normal human subjects and in chronic obstructive pulmonary disease (COPD) patients, but a small bronchodilator effect in asthmatic patients (178). NO-dependent airway relaxation is partially due to activation of $K_{c a}$ channels via guanylyl cyclase and PKG (461). Moreover, these relaxations are due to inhibition of $Ca^{2+}$ release, after stimulation of inositol trisphosphate receptors and ryanodine receptors, from sarcoplasmic reticulum of airway smooth muscle cells mediated via cGMP-dependent mechanisms (214).

Interestingly, there is increasing evidence for another mechanism, in addition to guanylyl cyclase activation, by which NO relaxes human bronchial smooth muscle (24, 127, 199, 341, 421). One of the metabolic pathways for NO also involves its reaction in the presence of thiol to form SNOs (126). SNOs are present in the airways of normal human subjects and in concentrations sufficient to influence airway tone and have a substantially greater half-life than NO (126). Recently, it has been found that severe asthma is associated with low concentrations of airway SNO, suggesting that the deficiency of such an endogenous bronchodilator mechanism is due to an accelerated degradation of SNO in the lungs of severe asthmatic individuals contributing to severe and refractory bronchospasm (88, 99, 133). Perkins et al. (341) showed that nitrosothiol-induced relaxation is mainly due to cGMP-independent component mediated by reversible oxidation of thiols on unspecified proteins that regulate contraction. Moreover, it has been demonstrated that the activation of $K_{c a}$ channels mediates part of NO-induced airway smooth muscle relaxation. NO donor-induced relaxation appeared to result in part from a direct cGMP-independent activation of $K_{c a}$ channels by NO, involving trans-nitrosylation reaction that could change the gating of the $K_{c a}$ channel (1). In a recent study it has also been found in canine tracheal smooth muscle contracted with KCl that GSNO decreases $Ca^{2+}$ sensitivity by affecting the level of regulatory myosin light-chain phosphorylation. This suggests that myosin light-chain kinase is inhibited or that smooth muscle protein phosphatases are activated by GSNO (328). Furthermore, it has been shown that SNO produced a concentration-dependent decrease in ADP-ribose cyclase, a regulatory enzyme of $[Ca^{2+}]_i$ in smooth muscle, through a cGMP-independent pathway involving trans-nitrosylation mechanisms (445). Finally, it has been examined whether two redox forms of NO, $NO^+$ (liberated by S-nitroso-N-acetylpenicillamine) and $NO^-$ (liberated by 3-morpholinosydnonimine) influence the cytosolic concentration of $Ca^{2+}$ and tone of human main stem bronchi. The authors found that $NO^+$ causes release of internal $Ca^{2+}$ in a cGMP-independent fashion, leading to activation of $Ca^{2+}$-dependent $K^+$ channels and relaxations, whereas $NO^-$ relaxes the airways through a cGMP-dependent and $Ca^{2+}$-independent pathway (200). In conclusion, the endogenous release of NO as well as the exogenous application of NO donors appear to activate several molecular mechanisms that synergically induce airway smooth muscle relaxation.

**E. NO Against Airway Smooth Muscle Contraction**

1. **In vivo studies**

Endogenous NO is also able to modulate excitatory airway responses induced by different mediators in animal models. Nijkamp et al. (315) showed in guinea pigs that aerosolized NOS inhibitors enhanced bronchoconstriction induced by increasing intravenous doses of histamine in vivo, suggesting a modulator role for endogenous NO in airway reactivity. Furthermore, Ricciardolo et al. (366) found a l-arginine/NO-dependent modulation of bradykinin-induced bronchoconstriction in guinea pigs that originates independently from the simultaneous activation of the excitatory neural component: postganglionic cholinergic nerves and capsaicin-sensitive afferent nerves (366). The latter group of investigators also noted that acid inhalation in guinea pigs stimulates a tachykinin- and bradykinin-mediated bronchoconstriction that is limited by endogenous release of NO (367). The NK$_1$ receptor is likely to be responsible for bronchoprotective NO release in the airways after tachykinin stimulation (370). Interestingly, bronchoconstriction provoked by stimulation of protease activated receptor-2 (PAR-2), after intracheal instillation or intravenous injection of trypsin or the tethered ligands for PAR-2, was inhibited by tachykinin antagonists and potentiated by NOS inhibitor (308). Furthermore, it has been shown that eNOS$^{-/-}$ mice were more hyperresponsive to inhaled methacholine and less sensitive to NOS inhibitor compared with wild-type mice, demonstrating that NO derived from eNOS plays a physiological role in controlling airway reactivity (100). In a recent study airway hyperresponsiveness to methacholine was completely abolished in eNOS-overexpressing, ovalbumin-challenged mice compared with control mice in conjunction with a decrease in the number of lymphocytes and eosinophils in the bronchoalveolar lavage fluid (416). In contrast to eNOS it has also been postulated that in mice nNOS could have a role in promoting airway hyperresponsiveness (74, 75).
Different groups of investigators have shown that acute bronchoconstriction induced by allergen inhalation is potentiated by NOS inhibitors in sensitized guinea pigs in vivo, suggesting a modulation by endogenous protective NO on early asthmatic reaction in animal model (286, 342, 343). Other in vivo studies in guinea pigs have shown that the enhanced airway reactivity induced by allergen (6 h after exposure) is not further potentiated by pretreatment with NOS inhibitors (393, 394) and that virus-induced airway reactivity is completely blocked by low doses of inhaled l-arginine (112), suggesting that allergen- or virus-induced airway hyperreactivity is due to the impairment of endogenous release of protective NO. More specifically, it can be postulated that a deficiency in eNOS-derived NO contributes to the increased airway reactivity after early response (EAR) to allergen (4–6 h), whilst a recovery in iNOS-derived NO production aids the reversal of airway reactivity after the late response (LAR: 24–48 h) in guinea pigs. This is supposed by the lack of effect of the specific iNOS inhibitor aminoguanidine on airway reactivity to histamine after EAR and by a significant potentiation of the partially reduced airway reactivity to histamine after the LAR induced by inhalation of the specific iNOS inhibitor aminoguanidine (393). More recently, it has been noted that expression of NOS I is reduced at 6 h, but not at 24 h, after allergen challenge in association with a decrease in constitutive NOS activity and in the amounts of exhaled NO. Together with maximal airway hyperresponsiveness to histamine, this suggests that the transient downregulated NOS I may have a role in airway hyperresponsiveness (387). In agreement with the previous studies, Toward and Broadley (423) found that exposure to inhaled LPS initially inhibited NO synthesis and the reduced NO levels coincided with the period of increased airway reactivity to histamine (1 h after exposure) in guinea pig. In contrast, 48 h 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 a renewal of NO synthesis probably derived by cytokine-induced NFKB activation of iNOS gene (265), with a bronchial relaxant effect.

For in vivo studies in humans, the reader is referred to section vB.

2. In vitro studies

Bradykinin, endothelin-1, substance P, adenosine, and calcitonin-gene related peptide, applied to the inside of intact tracheal tubes, provoke concentration-dependent relaxations (9, 93, 101–103, 316). The relaxations are 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 (9, 93, 101–103, 316). This effect was mimicked by removal of airway epithelium (111), suggesting that airway epithelium releases NO, which counteracts smooth muscle contraction induced by different spasmodens (9, 93, 101–103, 316). These striking results demonstrate the functional importance of epithelium in airway reactivity, not merely considered as a physical protective barrier between spasmodens and smooth muscle but as a modulator of bronchomotor tone via the release of relaxant substances (so-called epithelium-derived relaxing factors). Treatment of guinea pig trachea in vitro with an inactivator of guanylyl cyclase caused a fivefold increase in the sensitivity to histamine contractile response, indicating the involvement of NO/cGMP pathway in the development of airway hyperresponsiveness (385). Moreover, alterations in guanylyl cyclase activity may account for the strain-related differences in airway reactivity in rats (195). A further study showed that the electrochemical detection of bradykinin-induced NO release in guinea pig airways was fast (duration ~2 s), mainly dependent on the epithelium and absent in Ca2+-free medium, suggesting that a Ca2+-dependent eNOS pathway seems to be involved in the endogenous release of bronchoprotective NO (Fig. 1) (371).

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 raises significantly cGMP levels in guinea pig airways and that this effect is blocked by the pretreatment with NOS inhibitors and in epithelium-denuded preparations. This suggests that cGMP is the final mediator of the bronchoprotection dependent on epithelium-derived NO in this species (102). Meurs et al. (291) demonstrated that polycation-induced airway hyperreactivity to methacholine is dependent on the deficiency of endogenous NO, suggesting that polycationic peptides released by activated eosinophils in the inflamed airways may contribute to the deficiency of bronchoprotective eNOS-derived NO. In a further study these authors found that endogenous arginine activity potentiates methacholine-induced airway constriction by inhibition of NO production in naive guinea pig, presumably by competition with eNOS for the common substrate l-arginine (288). In a recent and elegant study, Ten Broeke et al. (417) showed that calcium-like peptides (CALP1 and CALP2) targeting calcium binding EF hand motif of calcium sensors (calmodulin and calcium channels) may have a role in regulating airway responsiveness by controlling [Ca2+]i, and, consequently, modulating the activity of eNOS (Fig. 1) (417). In fact, they observed that CALP2 inhibition of CALP1-induced airway hyperresponsiveness was Ca2+ epithelium dependent and NO mediated (417). Interestingly, they found that bradykinin-induced [Ca2+]i increase in epithelial cells was markedly higher after incubation with CALP2. In allergen-challenged guinea pigs,
the enhanced contractile response to agonists in tracheal preparations after early reaction was not augmented by NOS inhibition as shown in naive animals, suggesting an impairment of protective NO (70). In a further study the same authors showed that L-arginine administration reduced methacholine-induced contraction in isolated perfused tracheas from guinea pigs, indicating that limitation of the substrate may underline the reduced eNOS activity and the excessive contractile response (69). Finally, it has also been demonstrated that increased arginase activity contributes to allergen-induced deficiency of eNOS-derived NO and airway hyperresponsiveness after early allergen reaction in guinea pigs, presumably by direct competition with eNOS for L-arginine (290).

F. NO and Pulmonary-Bronchial Circulations

1. NO and pulmonary circulation

Nitrogen oxides can account for the biological activity of EDRF and are involved in the regulation of vascular tone (189, 257). Release of NO from endothelial cells in the pulmonary circulation appears to regulate vascular basal tone and counteract hypoxic vasoconstriction (Fig. 1) (344). Furthermore, NO release is apparently decreased in chronic hypoxia (4). Intravenous infusion of the NOS inhibitor L-NMMA increases pulmonary vascular resistance in normal adults pointing towards a role for endogenous NO in the control of pulmonary vascular tone at baseline (65). In the healthy human, eNOS isoform is present in the endothelium of pulmonary vessels, but its expression is downregulated in patients with primary pulmonary hypertension (136). This suggest that the pulmonary vasocstriction and the increased smooth muscle layer in the pulmonary vessels, main features of this disease, are associated with impaired expression of eNOS. Interestingly, these abnormalities might be associated with smoking. In a pig model challenge, unfiltered cigarette smoke induced variable responses in the pulmonary circulation, whereas inhalation of filtered smoke caused rapid and consistent pulmonary vasodilation, probably NO mediated (11). An in vitro study of pulmonary artery endothelial cells incubated with cigarette smoke extract resulted in a time- and dose-dependent decrease in eNOS activity associated with a nonreversible reduction of eNOS protein content and eNOS mRNA. This indicates that chronic exposure of cigarette smoke may contribute to the risk of pulmonary endothelial dysfunction via impairment of eNOS expression (409).

Impaired release of endothelium-derived NO from pulmonary vessels has also been observed in patients with COPD and cystic fibrosis (79). Moreover, isolated pulmonary arteries of patients undergoing heart-lung transplantation for end-stage chronic lung diseases have impaired endothelium-dependent relaxation (67). Recently, it has been demonstrated that overproduction of eNOS-derived NO can inhibit not only the increase in right ventricular systolic pressure associated with pulmonary hypertension, but also remodeling of the pulmonary vasculature and right ventricular hypertrophy induced by chronic hypoxia (Fig. 1) (327). In addition, the lungs of caveolin-1 knockout mice displayed thickening of alveolar septa caused by uncontrolled endothelial cell proliferation and fibrosis, suggesting an important role for caveolin-1 in endothelium-dependent relaxation of pulmonary vasculature (82). Polymorphisms of the eNOS gene have been associated with high-altitude pulmonary edema, suggesting that a genetic background may underlie the impaired NO synthesis in the pulmonary circulation of this disease contributing to its exaggerated pulmonary hypertension (83).

Interestingly, recent evidence suggests ethyl nitrite is more potent as a selective pulmonary vasodilator in humans and other mammals, and is associated with less withdrawal rebound hypertension, than NO itself (306, 307). This is important because ethyl nitrite is a potent S-nitrosylating agent that releases relatively little NO gas. Consistent with recent observations of Gow et al. (144), this observation suggests that the most relevant reaction leading to pulmonary vascular smooth muscle relaxation may involve S-nitrosylation chemistry.

2. NO and bronchial circulation

Of note, endogenous NO regulates basal bronchial vascular tone, and exogenous NO accounts for most of the bronchial vasodilatation observed after inhalation of cigarette smoke (11). The airway vasculature has also been shown to dilate in vivo when animals are ventilated with NO (59). Finally, endogenous endothelial NO significantly influences acetylcholine-induced bronchovascular dilation (389), but not the vagally induced bronchial vascular dilation in sheep (23).

Conflicting results have been reported about the role of endogenous NO in vascular permeability (247). A recent study in guinea pigs demonstrated 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 (211). The authors also showed that the substance P- and LTD₄-induced rise in plasma extravasation 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. It has also been shown that allergen inhalation in sensitized guinea pigs caused microvascular leakage in all airway portions which was suppressed in a dose-dependent manner by pretreatment with the NOS inhibitor L-NAME, suggesting that endogenous NO increases airway microvascular leakage after airway allergic reaction (295). Similar results have been found after administration of LPS, which was able to provoke a significant plasma leakage in rat trachea inhibited by the NOS inhibitor L-NAME. This effect was paralleled by an increase in iNOS activity in LPS animals, suggesting that iNOS-derived NO is responsible for LPS-induced increase in plasma leakage (33). On the contrary, these authors found that in the trachea of vehicle-treated rats L-NAME significantly increased plasma leakage, suggesting an inhibitor role of NO on plasma leakage under physiological conditions. Thus the possibility that alteration of bronchial blood flow by NOS inhibitors confounds the results on plasma leakage cannot be excluded. Further studies examining blood flow through individual microvascular beds would permit greater information about the precise role of endogenous NO on this important aspect of airway microcirculation relevant to disease such as asthma.

G. NO and Mucus-Electrolyte Secretions in the Airways

NOS inhibitors did not affect mucus glycoprotein secretion tonically, but significantly reduced both methacholine- and bradykinin-induced secretion from feline tracheal isolated submucosal glands (312). In addition, NO generator isosorbide dinitrate significantly increased submucosal gland secretion. Taken together, these results suggest that endogenous NO stimulates airway submucosal gland secretion. Taken as a whole, together these data suggest that NO inhibitors confounds the results on plasma leakage cannot be excluded. Further studies examining blood flow through individual microvascular beds would permit greater information about the precise role of endogenous NO on this important aspect of airway microcirculation relevant to disease such as asthma.

Abnormal electrolyte transport produces changes in airway surface liquid volume and composition, inhibits mucociliary clearance, and leads to chronic infection of the airways, as occurs in cystic fibrosis. Modulation of ion channels by NO has emerged recently as a significant determinant of ion channel function (87). NO activates both apical anion channels and basolateral potassium channels via cGMP-dependent pathway (86). Thus NO is a physiological regulator of transepithelial ion movement, and alterations of its generation and action may play an important role in the pathogenesis of lung disorders characterized by hypersecretion of airway surface liquid.

Of note, SNOs have several established effects of potential benefit in the cystic fibrosis airway. These include ventilation-perfusion matching, smooth muscle relaxation, increased ciliary beat frequency, inhibition of amiloride-sensitive sodium transport, augmentation of calcium-dependent chloride transport, augmentation of neutrophil apoptosis, and antimicrobial effects as recently reviewed (403). Additionally, recent evidence suggests that physiological levels of SNOs can increase the expression, maturation, and function of ΔF508 mutant CFTR protein, apparently through S-nitrosylation of trafficking proteins involved in the ubiquitination and degradation of the molecule (14, 179, 466). In this regard, it is of particular interest that metabolism of SNOs appears to be accelerated in the cystic fibrosis airway and that SNO levels are nearly undetectable in the bronchoalveolar lavage fluid of patients with mild cystic fibrosis (146). Augmentation of SNO levels by therapeutic administration of GSNO appears to be well-tolerated in patients with cystic fibrosis and to lead to an improvement in oxygenation (403). Of note, inhaled NO does not improve oxygenation in these patients (360).

III. NITRIC OXIDE AND OXIDATIVE STRESS: “NITROSATIVE STRESS”

Reactive oxygen species (ROS) are generated by various enzymatic reactions and chemical processes or they can be directly inhaled. NO can interact with ROS to form other reactive nitrogen species (RNS) (Figs. 2 and 3). ROS, NO, and RNS are essential in many physiological reactions and are important for the killing of invading microorganisms (Fig. 2). However, when airway cells and tissues are exposed to oxidative stress elicited by environmental pollutants, infections, inflammatory reactions, or decreased levels of antioxidants, enhanced levels of ROS and RNS can have a variety of deleterious effects within the airways, thereby inducing several pathophysiological conditions (Fig. 3). ROS and RNS can damage...
DNA, lipids, proteins, and carbohydrates leading to impaired cellular functions and enhanced inflammatory reactions (Figs. 2 and 3). In this way, ROS and RNS play a prominent role in the pathogenesis of various lung disorders such as adult respiratory distress syndrome (ARDS), interstitial lung disease, cystic fibrosis, COPD, and asthma (37, 110, 141, 182, 362, 431).

**A. Formation of RNS**

Because NO and superoxide are free radicals, both molecules rapidly react with many different molecules in a biological environment. Of particular interest is the interaction between the two molecules and their reactive downstream metabolites. Enhanced cytotoxicity is possible when NO and superoxide are released simultaneously, which is a likely event during inflammatory responses (Fig. 2). For example, the efficient killing of *Salmonella* by murine macrophages is dependent on both NADPH oxidase-derived superoxide and iNOS-derived NO. Many of the products formed by the interaction of superoxide and NO are even more reactive than their precursors. The most direct interaction between NO and superoxide is their rapid isostoiichiometric reaction to form the potent oxidant peroxynitrite (ONOO•). Peroxynitrite induces the formation of nitrotyrosine residues; however, tyrosine nitration may also be found after exposure of proteins to nitrite (NO2−) in association with hypochlorous acid (HOCl) and myeloperoxidase (MPO) or eosinophil peroxidase (EPO). As mentioned in the different sections in the text, high concentrations of NO formed by iNOS, peroxynitrite, and tyrosine nitration may all cause a variety of pathophysiological effects.

**FIG. 3.** Schematic overview of how inhaled substances or proinflammatory mediators contribute to the production of reactive oxygen and nitrogen species in the airways that will finally result in pathophysiological effects. Upon appropriate stimulation, inflammatory cells and a number of airway resident cells can generate superoxide (O2•−) via activation of NADPH oxidase or form high amounts of nitric oxide (NO) via an increased expression of iNOS. NO reacts with superoxide to form the potent oxidant peroxynitrite (ONOO•). Peroxynitrite induces the formation of nitrotyrosine residues; however, tyrosine nitration may also be found after exposure of proteins to nitrite (NO2−) in association with hypochlorous acid (HOCl) and myeloperoxidase (MPO) or eosinophil peroxidase (EPO). As mentioned in the different sections in the text, high concentrations of NO formed by iNOS, peroxynitrite, and tyrosine nitration may all cause a variety of pathophysiological effects.
the formation of ROS. These compounds can either spontaneously or enzymatically dismutate to hydrogen peroxide. Granulocytes contain peroxidases (myeloperoxidase and eosinophil peroxidase) that are able to catalyze the reaction of hydrogen peroxide with halides leading to the formation of hypohalides (e.g., hypochlorous acid, Fig. 3) (22, 240).

Formation of ROS takes place constantly in every cell during normal metabolic processes. Cellular sites for production of ROS include mitochondria, microsomes, and enzymes (e.g., xanthine oxidase, P-450 monooxygenase, cyclooxygenase, lipoxygenase, indole amine dioxygenase, monoamine oxidase) (120, 431). Activated phagocytic cells (neutrophils, eosinophils, monocytes, and macrophages) produce large amounts of ROS. These cells are stimulated when encountering inhaled particles, microorganisms, or other mediators that lead to the activation of the membrane-bound NADPH-oxidase complex and the generation of the superoxide anion (21, 22, 167). Compounds of this enzyme complex have also been found to be present in other cell types such as vascular smooth muscle cells and endothelial cells (205, 279).

NO is a radical molecule that is formed by a wide range of cells, including nerves, (activated) macrophages, fibroblasts, airway and vascular smooth muscle cells, endothelial cells, and epithelial cells (110, 308, 384). In contrast to murine macrophages, it was found that human mononuclear phagocytes did not release large amounts of NO, despite the presence of iNOS (309, 444). However, the lack of NO synthesis in these experiments is probably an in vitro artifact. Adequate stimulation in vivo will lead to NO release by human macrophages (98) and probably cellular interactions (e.g., with airway epithelial cells) and/or local production of regulatory factors are of importance for the NO production (338).

Besides the generation of reactive species via cellular pathways, formation of ROS and RNS in the lungs can also take place after inhalation of exogenous compounds like ozone, nitrogen dioxide, cigarette smoke and other chemicals, and dust particles (246, 431). In addition, such exposures lead to depletion of endogenous antioxidants that are present in the epithelial lining fluid.

Due to the complex chemistry and often short half-life of RNS, the exact metabolic fate in vivo remains unclear. Furthermore, it is almost impossible to attribute a given effect in vivo to a certain reactive intermediate. Nonetheless, some stable end products of RNS are detectable in body fluids and tissues. First, NO decomposes into nitrite and nitrate, and these metabolites can be measured in plasma (222). Furthermore, 3-nitrotyrosine residues have been found in tissue samples by the use of immunohistochemistry (386), but also in biological fluids (322). However, it is often difficult to interpret results from these kinds of experiments since there is a high risk of artifacts. 3-Nitrotyrosine is readily formed by a NO-dependent process mediated by myeloperoxidase, with hydrogen peroxide and nitrite as substrates (Fig. 3) (89, 226). Moreover, eosinophil peroxidase is an even stronger promoter of 3-nitrotyrosine formation via this pathway (Fig. 3) (310, 454). At present, the relative contribution of these peroxidase-mediated pathways and peroxynitrite to in vivo 3-nitrotyrosine formation is the subject of debate (90, 361).

Nitrite and nitrate levels in plasma, for example, can reflect the dietary intake rather than NO metabolism in vivo (6). Moreover, NO is also formed enzyme-independently from nitrite under acidic conditions (471). Recently, Hunt et al. (185) showed that the pH in the airways drops dramatically during an acute asthma attack, which facilitates the conversion of nitrite to NO. Hence, increased NO concentrations in the exhaled air of asthmatic patients may reflect nitrite conversion rather than NOS activity.

Enzymes and chemicals are present within the airway cells and in the airway epithelial lining fluid to protect against the toxicity of generated ROS and RNS. The major enzymatic systems present in the airways are manganese and copper-zinc superoxide dismutases, which rapidly convert the superoxide anion to hydrogen peroxide, catalase that converts hydrogen peroxide into oxygen and water, and the glutathione redox system (GSH-peroxidase and GSH-reductase) that inactivates NO, hydrogen peroxide, and other hydroperoxides (17, 53, 96, 237, 348, 362). The epithelial lining fluid of the respiratory tract contains large amounts of glutathione, and >95% of this glutathione is in the reduced form (54). Moreover, thiol groups in proteins can bind NO. Other nonenzymatic factors with scavenging properties for oxygen radicals that can be present within the airways are vitamin E (a-tocopherol), vitamin C (ascorbic acid), uric acid, β-carotene, flavonoids, taurine, lactoferrin, albumin, and bilirubin. A disadvantage of limiting RNS formation is of course a compromised defense against invading microorganisms. Moreover, nonspecific NOS inhibition may lead to a compromised function of NO as a paracrine messenger, for instance, leading to hypertension (411). The successful use of NOS inhibition is therefore dependent on the isoform of NOS involved, and on the selectivity of the inhibitor used. Nonetheless, limiting superoxide production by NADPH oxidase is of particular interest, since superoxide release is also required for the formation of many RNS, and inhibition of NADPH oxidase should not compromise other NO functions.

B. Airway Damage by “Nitrosative Stress”

The effects of RNS, once formed in vivo, on tissues, cells, and biomolecules are diverse. Important targets of RNS in proteins are, for example, tyrosine residues (432),...
The levels of NO in the exhaled air are determined by exhaled NO, both for adults and in children (13, 26, 227). National guidelines on the methods of measurement of standardization has recently been accomplished by inter-questions carefully standardization of the measurement. Such critically dependent on expiratory exhalation analyzers (156). The measurement of exhaled NO is of NO, in the ppb range as measured by chemiluminescence analyzers (88, 133, 403); and SNO catabolic enzymes (125), and heme groups (97). Furthermore, RNS are able to compromise cell function. Exposure of cells to RNS leads to both apoptosis and necrosis dependant on the severity of cell damage (310). In a recent study it was demonstrated that MAP kinase may mediate signal transduction pathways induced by reactive nitrogen in lung epithelial cells leading to cell death (311). Again, these detrimental effects may affect both an invading pathogen and the (infected) host (Fig. 2).

IV. EXHALED NITRIC OXIDE

Exhaled air of humans contains detectable amounts of NO, in the ppb range as measured by chemiluminescence analyzers (156). The measurement of exhaled NO is critically dependent on expiratory flow (346), which requires careful standardization of the measurement. Such standardization has recently been accomplished by international guidelines on the methods of measurement of exhaled NO, both for adults and in children (13, 26, 227). The levels of NO in the exhaled air are determined by 1) NO production by various cells in the airways and/or lung parenchyma, 2) diffusion of NO into the capillary circulation, and 3) alveolar ventilation and bronchial flow (187).

Exhaled NO production by the airways and lung parenchyma, in turn, appears to be determined by 1) the activity of all three NO synthase (NOS) isoforms, but particularly isoforms I and II (75, 443); 2) the activity of arginase 2 and metabolic enzymes that regulate the endogenous NOS inhibitor asymmetric dimethyl arginine (313); 3) prokaryotic, denitrifying species colonizing the upper and lower airways (131); 4) SNO catabolic enzymes (88, 133, 403); and 5) processes/enzymes that regulate airway pH and nitrite reduction, such as glutaminase (184).

It appears that the NO production and exhalation NO concentrations can be predicted by a two-compartment model of the lung, consisting of a nonexpansible compartment representing the conducting airways and an expandable compartment representing the respiratory bronchioles and alveoli. The model predicts that both compartments contribute to NO in the exhaled breath and that the relative contributions of airways and parenchyma can be separated by analysis of the relationship between exhaled NO output (nl/s) against expiratory flow rate (ml/s) (410, 425). Interestingly, such analysis may indeed allow the discrimination of airway diseases, such as asthma, from alveolitis (255) or liver cirrhosis (72) in patients with elevated levels of exhaled NO. This suggests that exhaled NO might be used in differential diagnoses, based on recent theoretical and experimental physiology.

A. Exhaled NO and Bronchial Asthma

Patients with atopic asthma show increased levels of exhaled NO compared with healthy controls (148, 236). In asthma, the increased levels of exhaled NO have a predominant lower airway origin (229, 282) and appear to be associated with increased expression of corticosteroid-sensitive iNOS (386). However, there is recent evidence that exhaled NO levels in asthma are also associated with a known functional missense sequence variant in the eNOS gene (G894T) (407). This indicates that both NOS II and NOS III are important in determining the NO detected in the exhaled air in patients with asthma. Furthermore, exhaled NO may reflect disease severity (234) and, to a greater extent, clinical control of asthma (402) particularly during exacerbations (71, 231, 281).

Exhaled NO has been used to monitor asthma exacerbations, both spontaneous (281) and induced by steroid reduction (235), and the effect of anti-inflammatory treatment in asthma (234). It can be postulated that asthma treatment with corticosteroids results in a reduction of expired NO levels due to both 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) (228) and dose-dependent reduction (203, 433). Since already low doses of inhaled steroids (400 µg budesonide) seem to be sufficient to reduce elevated exhaled NO levels to normal values in patients with intermittent or mild persistent asthma (203), the question arises whether these low NO levels indeed reflect optimal control of the underlying airways inflammation or just switching off of expression of iNOS or of a pH regulatory enzyme such as glutaminase (184). In patients with more severe persistent asthma, airway inflammatory processes may overcome this steroid sensitivity of NO, leading to increased levels of exhaled NO even during treatment with high doses of oral or inhaled corticosteroids (235).

During the last few years several studies have been performed to assess the relationship between levels of exhaled NO and lung function parameters or other markers of airway inflammation. Exhaled NO in patients with asthma is correlated with airway hyperresponsiveness to methacholine (84, 204), as well as peak flow variability (260). Furthermore, exhaled NO is associated with eosinophilic inflammation as determined in blood (401), urine (283), bronchoalveolar lavage (260), and sputum (137) in...
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asthmatics with varying disease severity. Recently, a significant relationship has also been shown between exhaled NO and mucosal eosinophil numbers in bronchial biopsies from children with difficult asthma (336) and from atopic adult asthmatics after allergen challenge (364). This indicates that exhaled NO is a novel noninvasive biomarker reflecting airway eosinophilic inflammation in asthma. High production of endogenous NO such as in acute asthma may result in a deleterious effect and may be involved in the orchestration of eosinophilic inflammation that characterizes asthma.

B. Exhaled NO and Other Respiratory Disorders

Exhaled NO levels in COPD are conflictual (61, 66, 284, 383), but it seems that smoking habits and disease severity are the most important factors influencing exhaled NO levels in these patients (406). Current smokers (232) and severe COPD (particularly in combination with cor pulmonale) (62) show lower levels of exhaled NO than ex-smokers and mild-moderate COPD. Increased exhaled NO levels have been reported in hospitalized patients during an exacerbation of COPD (5). Interestingly, exhaled NO levels returned to control values only months after discharge of those steroid-treated patients, suggesting different inflammatory mechanisms in COPD compared with the highly steroid-sensitive asthmatics (5).

Acidosis, a feature of acute respiratory failure frequently associated with exacerbations of COPD, may also increase the release of NO (185). Moreover, pH is low in exhaled breath condensate during inflammatory diseases (245). Other disorders associated with increased exhaled NO levels include rhinitis (168), bronchiectasis (233), active pulmonary sarcoidosis (300), active fibrosing alveolitis (332), and acute lung allograft rejection (400). In contrast, low levels of exhaled NO have been reported in patients with PCD (215), cystic fibrosis (80, 147), PiZZ genotype-related α1-antitrypsin deficiency (275), and pulmonary hypertension (372). Certain pulmonary infections, such as viral respiratory illnesses, increase exhaled NO values (25), while others, such as chronic colonization of the cystic fibrosis airway with denitrifying organisms, attenuate exhaled NO values (131).

In particular, PCD, including Kartagener’s syndrome, is a genetic disease characterized by defective motility of cilia, in which the levels of exhaled NO are very low compared with normal subjects. Such low levels of exhaled and nasal NO are not seen in any other condition and are therefore used as a screening procedure to detect PCD among patients with recurrent chest infections or male infertility caused by immotile spermatozoa (47). The mechanism of low NO production by nasal and airway mucosa in PCD is unknown, but it might be linked to genetic abnormalities in iNOS gene expression as in cystic fibrosis (81).

V. NITRIC OXIDE AND PATHOPHYSIOLOGY OF THE RESPIRATORY SYSTEM

A. NO and Immune-Inflammatory Responses in the Airways

1. NO and cytokine networks

In 1991, Jorens et al. (206) showed that pulmonary rat macrophages, alveolar as well as pleural, can produce L-arginine-derived nitrite in a dose- and time-dependent manner, after activation with endotoxin, rat recombinant IFN-γ and opsonized zymosan in vitro (206). The authors found that glucocorticoids blocked the induction of nitrite in alveolar macrophages by all of the stimuli mentioned above (206). These results suggest that part of the anti-inflammatory effect and immunosuppressive effects of glucocorticoids are due to their inhibition of the induction of iNOS. During inflammatory responses a variety of cytokines are expressed and released in the lung and airways. The cytokine network may play an important role in the modulation of inflammation in the local environment. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and muramyl dipeptide, a constituent of the bacterial wall, are able to enhance IFN-γ-induced nitrite production in rat alveolar macrophages in vitro, with GM-CSF serving as a priming factor (207). In addition to alveolar macrophages, rat lung fibroblasts are capable of producing nitrite upon stimulation with IFN-γ. This effect is markedly enhanced in fibroblasts after incubation with endotoxin and IL-1β, suggesting that IL-1β is an efficient priming signal for IFN-γ-induced nitrite production (208). In contrast to NO-inducing cytokines, other cytokines such as transforming growth factor-β, IL-4, and IL-10 have been shown to inhibit the expression of iNOS (314). Recombinant human IL-11 is able to reduce IL-12-induced IFN-γ production and to enhance IL-4 and IL-10 production modulating cytokine production in CD4+ cells with the subsequent reduction in NO production from macrophages (38). Recently, it has been shown that NO inhibited LPS-stimulated inflammatory cytokine production (TNF, IL-1, and MIP-1α), but not basai cytokine levels, by normal human alveolar macrophage, suggesting a modulatory role for NO in proinflammatory cytokine secretion by normal human alveolar macrophage (420).

2. NO and T cells

NO may play a role in nonspecific defense mechanisms against pathogens and may be involved in the signaling between macrophages and T cells (28). CD4+ T helper (Th) cells are important in host defense and have been implicated in chronic inflammatory diseases. Two types of Th cell are differentiated by the pattern of cyto-
kines secreted on activation. Th1 cells release IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-10 (305, 321). These patterns of cytokine production largely determine the effector functions of the two subsets of T cells (378). Th1 cells produce IFN-γ that activates macrophages to produce NO and kill pathogens (294). Inhibition of NO production by analogs of L-arginine results in inactivation responses to different stimuli involves NO release by inflammatory cells. Thus NO seems to exert a self-regulatory effect on cellular activity and suggest that inhibi-tors of this molecule may represent a worthwhile therapeutic strategy for allergic diseases including asthma.

In addition, NO has been reported to promote the production of chemotactic factors (chemokines) for eosinophils in mice (424), suggesting the possibility that NO acts as part of a positive-feedback loop in which inflammatory cells produce NO and thereby promote their further recruitment through the action of chemokines.

Recent studies also demonstrated that NO inhibits macrophage-derived IL-12 release, which is a major inducer of Th1 cells, preventing the excessive amplification of Th1 cells (180), and that NO-generating agents increased the secretion of IL-4 in Th2 clones (58). This suggests 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.

B. NO and Airway Hyperresponsiveness

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. Clinical researchers investigated the capability of endogenous NO to affect AHR in asthma. Ricciardolo et al. (365), for the first time, performed a randomized double-blind placebo-controlled study of the effect of NOS inhibition in bradykinin-induced asthma. The authors described a 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 brady-kinin compared with methacholine, indicating that a mediator-specific response is involved. In a further study, the same group revealed an impairment of NO synthesis inhibition on AHR to bradykinin in severe asthma, possibly...
due to the reduction or absence of eNOS in the airway of severe asthmatic patients (363). Following these observations, it has also been discovered that severe asthmatics treated with higher dose of corticosteroids than in the previous study are less hyperresponsive to bradykinin, but that the pretreatment with NOS inhibitor markedly enhanced AHR to bradykinin as shown in mild asthma (34). This suggests an effect of high doses of corticosteroids in renewing eNOS activity by suppression of iNOS expression. A significant potentiation of NOS inhibitors has also been found in AHR to AMP and histamine, but not to allergen-induced bronchoconstriction in asthmatics (412, 413).

Allergen and viral infection are also called inducers of airway reactivity since they are able to increase native reactivity in animal and human asthma (63, 109). A recent study showed that increased AHR to bradykinin, induced by allergen exposure in asthma, is due to impaired production of bronchoprotective NO, a phenomenon that is associated with downregulation of eNOS and upregulation of iNOS within the airway epithelium (369). The latter findings underscore the relevance of bronchoprotection by endogenous NO to limit AHR in asthma and warrant the development of treatment strategies to restore eNOS activity during exacerbations (369).

Recently, to examine the possible involvement of the eNOS gene as the genetic basis of bronchial asthma it has been investigated whether there was any association between bronchial asthma and polymorphisms of eNOS gene. The study by Lee et al. (253) revealed 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 different severities of asthma. In addition, as mentioned above, a recent analysis has demonstrated an association between a missense sequence variant in the eNOS gene and exhaled NO levels in asthma, in the absence of associations of this mutation with the level of airways obstruction or its reversibility in these patients (407). Therefore, all of these results suggest that polymorphisms of the eNOS gene may be associated with the development of asthma, but the severity of asthma may not be influenced by polymorphisms of eNOS gene.

C. NO and Cell Proliferation-Survival in the Airways

1. NO and airway remodeling

Airway smooth muscle hypertrophy and hyperplasia, features of airway remodeling, are important determinants of airway hyperresponsiveness in asthma. In vitro studies have recently demonstrated that DNA synthesis and proliferation of human airway smooth muscle cells (HASMC) are reduced by exogenous administration of NO donors (160, 334). More recently, it has been demonstrated that NO inhibited HASMC proliferation in G1 phase via cGMP-dependent pathway, but the inhibition of HASMC proliferation in S phase was due to cGMP-independent inhibition of ribonucleotide reductase (161). These newly discovered antiproliferative effects of NO on airway smooth muscle might become an important clue for future strategies to prevent airway remodeling in chronic asthma and COPD.

2. NO and posttransplant obliterative bronchiolitis

The main cause of mortality following lung transplantation is chronic rejection, manifested morphologically as obliterative bronchiolitis. It has been suggested that damage to the respiratory epithelium initiates proliferation of mesenchymal cells, leading to dense collagenous scarring in small airways. iNOS is strongly expressed in the damaged epithelium in human obliterative bronchiolitis, indicating NO as a mediator of epithelial destruction. Fibroblast proliferation is associated with changes in morphology of fibroblasts accompanied by alterations in iNOS expression. Taken together, these results suggest a dual role for NO in obliterative bronchiolitis following lung transplantation through destruction of epithelium and stimulation of fibroblast activity (379).

3. NO effects on apoptosis

Nitrogen oxides can promote either cell survival or cell death, depending on the chemical species, redox state, concentration, and target cell type. This paradox was first described by Lipton et al. (262) who showed that physiological concentrations of S-nitroso-L-cysteine protected against apoptotic and necrotic neuronal cell death (Fig. 1), while peroxynitrite in similar concentrations caused necrosis, in central nervous system neurons (Figs. 2 and 3). It has been observed that 250 nM (physiological concentrations) of S-nitrosocysteinyl glycine protects against eosinophilic apoptotic cell death (186). Recently, Nabeyrat et al. (311) showed that MAP kinases may mediate signal transduction pathways induced by peroxynitrite in lung epithelial cells leading to cell death.

Early studies on the effect of nitrogen oxides on cell survival were done with high micromolar or millimolar concentrations of “NO donors,” with the idea that NO radical was evolved from these species in nanomolar concentrations and was the only relevant nitrogen oxide. At these high concentrations (all >100 μM), SNos and NONOates augmented apoptosis in neutrophils (117), macrophages (287) and other leukocytes (43). Several mechanisms might be hypothesized, ranging from protein nitration to nitrosoamine-mediated cytidine to thymidine mutation (451). Of note, Meßmer et al. (287) have pro-
volved evidence that bcl-2 overexpression protects against this high-dose NO-induced apoptosis.

On the other hand, physiological concentration of SNOs protect against apoptosis. The mechanism appears to involve, at least in part, nitrosylation of the active-site cysteine of caspases 3 and 9. This effect was originally described in endothelial cells (176) and later characterized directly in lymphocytes (277). Indeed, there is now evidence that Fas-Fas-ligand binding leads to release of S-nitrosylated caspases from the mitochondrial intermembrane space into the cytosol, where these proteases are activated by denitrosylation, leading to apoptosis (278). This picture was recently clarified further by Haendeler et al. (158), who showed that endogenous or exogenous S-nitrosylation of thioredoxin cysteine-69 (which is not one of the redox active cysteines) dramatically increases the antioxidant activity of thioredoxin. This SNO-thioredoxin pool appears to be an important, if not the principal, cellular reservoir of SNO bioactivity. Thus physiological levels of NO/SNO may be antiapoptotic by augmenting antioxidant defenses and by increasing, through transnitrosation reactions, inactivation of caspasases. Although there is also evidence for cGMP-dependent up-regulation of bcl-2 in lymphocytes (134), it appears that S-nitrosylation/transnitrosation reactions represent the principal biochemical mechanism underlying the antiapoptotic effects of physiological levels of nitrogen oxides.

D. NO and Lung Cancer

The role of NO in cancer is multidimensional and based on timing, location, and concentration of the different nitrogen oxides. Several studies have implied that overexpression of NO in chronic inflammation can lead to genotoxicity, and thus NO is considered a tumor initiating agent (Fig. 2). NO may mediate DNA lesions via formation of carcinogenic nitrosamines, direct DNA mutations or DNA strand breaks by RNS and nitrosation inhibition of systems required to repair DNA lesions mediated by other genotoxic substances such as DNA alkyl transferase and DNA ligase (452). Cigarette smoking, the major cause of lung cancer, contains high concentrations of nitrogen oxides in the gas phase and other oxidants in the tar (465). Recently, it has been shown that incubation of plasmid DNA with extracts of cigarette tar and NO-releasing compound caused synergistic induction of DNA single-strand breakage. This suggest a genotoxic role of RNS, formed by the reaction between NO (gas phase) and ROS formed by autoxidation of polyhydroxyaromatic compounds of the tar, in lung cancer (465). Finally, a recent study noted that an excess of endogenously formed NO may induce a mutation of the p53 tumor suppressor gene containing mainly G:C-to-T:A transversion in the early stage of lung adenocarcinoma (123).

NO may also impact other stages of cancer development. These effects of NO are broad and often self-contradictory, spanning its involvement in cytostatic processes, cellular transformation, formation of neoplastic lesions, and regulation of various aspects of tumor biology (452). Thus NO may have both tumoricidal and tumor-promoting effects. NO has a cytostatic effect on tumor cells through inhibition of cellular respiration via modified activity of mitochondrial aconitase by nitration mechanisms and through inhibition of ribonucleotide reductase suppressing DNA synthesis (Fig. 1). Another possible consequence of NO production in cancer is apoptosis within the growing tumor, and this process has been implicated in the tumoricidal activity of NO (452). Furthermore, NO derived from leukocytes may have an antitumor role, and in particular monocyte-macrophage series have an important role in host surveillance against cancer (183). The cytotoxic/cytostatic activity of macrophages is, to a great extent, attributed to the upregulation of iNOS. In a recent study the mean fluorescent intensity of iNOS in alveolar macrophage (AM) from patients with primary lung cancer was increased compared with that from controls, and associated with increased exhaled NO levels. This indicates that in primary lung cancer the production of NO from AM is increased as a result of iNOS upregulation (263). Some reports further suggest that NO may contribute to suppression of metastasis. Kong et al. (243) have shown that NO inhibits tumor cell adhesion in a manner similar to the inhibition of leukocyte adhesion described for NO in ischemia-reperfusion injury (248). This suggests that low levels of NO produced by the endothelium will reduce metastasis to tissues such as the lung.

NO has been proposed to be an important mediator of tumor growth, and of note, NO could play an important role in tumor progression via regulation of angiogenesis. Enhanced angiogenesis can lead to accelerated growth of the primary tumor, as well as facilitating the process of metastasis (Fig. 3) (196). Angiogenesis is regulated by the cytokine vascular endothelial growth factor (VEGF) and modulated by a number of other cofactors, including TNF-α and transforming growth factor-β, which in part may also be regulated by NO in lung cancer (15). Paradoxically, other studies indicate that NO may downregulate angiogenesis via inhibition of the transcriptional regulation of the VEGF promoter (452). This paradox has been recently studied to characterize the direct effects of NO at the level of the tumor-endothelium interface with respect to angiogenesis using a Transwell two-compartment culture system with human endothelial cells and two human non-small-cell lung cancers (347). It has been found that baseline component of capillary formation at the endothelial-tumor interface is also NO dependent in line with other observations where endothelial-derived NOS is essential for angiogenesis. However, elevated con-
centrations of NO in endothelial-tumor microenvironment attenuate capillary formation via downregulation of matrix metalloproteinase activity and inhibition of protein tyrosine phosphorylation in the sprouting tips of nascent capillaries. The extent of inhibition depended on the concentration and flux of NO produced in this milieu (347). Another mechanism by which NO may promote tumor growth is by modulating the production of prostaglandins. In particular, NO increases the production of PGE₂, which may in turn increase the leakiness of tumor vasculature (452). On the other hand, PGE₂ also suppresses NO-dependent macrophage tumoricidal activity. Additionally, permeability of the tumor vasculature is mediated by NO produced by the tumor cells themselves and in turn may facilitate angiogenesis increasing tumor growth.

Finally, it has been found that total NOS activities and the intensity of NOS immunoreactivity are significantly higher in lung adenocarcinoma than those in other types of lung cancers, suggesting a specific role of NO in the metabolism and behavior of lung adenocarcinoma (122).

VI. INHALED NITRIC OXIDE

The purpose of this section is to evaluate the potential use and place of NO inhalation therapy in the treatment of diseases of the respiratory system. To appropriately examine this issue it is important to consider general problems due to the exposure of lung cells to NO in relation to dose and toxicity. Animal studies on the toxicity of inhaled NO (iNO) for up to 6 mo revealed no evidence of side effects using NO doses of <40 ppm (181, 319). Thus proposed treatments with iNO in humans vary from 2 to 36 ppm for periods of a few days to a few weeks (354, 381). NO solubilities from the Ostwald coefficient (448) give equilibrium concentrations of NO in extracellular fluid ranging from 3.2 to 58 nM in the absence of O₂. These concentrations are low, and the loss of NO by autoxidation would be negligible, since autoxidation is second order in NO (256). Thus the fate of iNO should be the following: 1) loss in exhaled air, 2) combination with oxyhemoglobin in erythrocytes, and 3) reaction with O₂ to form peroxynitrite. The first two possibilities do not exert toxic consequences.

1) In particular, we point out that the expired NO (eNO) values are 3 log orders lower compared with iNO values, suggesting an irrelevant physiological role for eNO.

2) Moreover, iNO reacts for its breakdown by interaction with oxygen and hemoglobin. The rate of the autoxidation with the formation of NO₂ increases exponentially with the concentration of both oxygen and NO (113). Thus the therapeutic efficacy of iNO may not rise dramatically with increased doses as the more NO given, the faster it is oxidized (238). In fact, higher doses of NO result in a relatively greater proportion of toxic products with little incremental yield of intact NO.

3) Finally, the reaction of NO with O₂⁻ is extremely rapid (182), and peroxynitrite (ONO₂⁻) is toxic at millimolar doses to all types (bacterial and mammalian) of cells. If O₂⁻ were in great excess to NO, the rate-limiting step in ONO₂⁻ formation would be diffusion of NO from air into solution. Thus ONO₂⁻ would approach the number of moles of NO in solution.

When administered as inhaled gas at low concentrations, NO diffuses into pulmonary vasculature of ventilated lung regions and selectively dilates the pulmonary vasculature (118, 121). iNO is distributed predominately to well-ventilated alveoli and not to collapsed or fluid-filled areas of the lung. Local vasodilation of well-ventilated lung regions will cause a “steal” of pulmonary artery blood flow toward well-ventilated alveoli, improving the matching of ventilation to perfusion and improving arterial oxygenation during acute lung injury. Systemic vasodilation does not occur because of the rapid binding and inactivation of NO by hemoglobin within the circulation (138). This effect is in contrast to that of intravenously administered conventional vasodilators (such as nitroprusside, nitroglycerin, or prostacyclin). These agents nonselectively dilate the pulmonary vasculature and augment blood flow to nonventilated areas, thereby increasing right-to-left shunting and reducing PaO₂.

The first pilot studies in humans have been performed by Higenbottam et al. (171) in 1988 demonstrating that iNO was able to reduce pulmonary hypertension in adult patients without major effects on the systemic circulation (339). A few years later, experiments in animal models revealed that iNO was also able to reverse hypoxic pulmonary vasoconstriction without impairing the pulmonary gas exchange (121, 349). Additionally, Roberts et al. (376) and Kinsella et al. (238) found that iNO might be useful in the therapy of the persistent pulmonary hypertension of the newborn (PPHN). In 1993, Rossaint et al. (381) revealed that both iNO (at doses of 18 and 36 ppm) and infused prostacyclin (4 ng · kg⁻¹ · min⁻¹) are able to reduce pulmonary resistance (~20% fall) in ARDS patients (381). In contrast to prostacyclin, which simultaneously caused systemic hypotension and decreased arterial oxygenation saturation, iNO did not induce any change in systemic hemodynamics, but improved arterial oxygenation significantly. Measurement of ventilation-to-perfusion ratio in these patients showed that intrapulmonary right-to-left shunting was increased by the infusion of prostacyclin but, in contrast, was reduced by iNO at 18 or 36 ppm due to redistribution of pulmonary blood flow toward areas with nearly normal ventilation-to-perfusion ratios. This study did not demonstrate any difference between the two doses of 18 or 36 ppm NO regarding pulmonary resistance and systemic oxygenation.
In ARDS patients, improvement of systemic oxygenation and reduction of pulmonary artery pressure are not correlated during NO dose-response studies. To explain this aspect, two different speculative theories (“diffusion theory” and “kinetic theory”) have been postulated. 1) NO quickly diffuses into tissue reaching a balance between the rate of diffusion and the rate of oxidation or binding to targets. Low doses of iNO probably induce diffusion only into vessels near ventilated alveoli (strictly selective vasodilation in ventilated areas) reducing intrapulmonary shunt areas and increasing systemic oxygenation. High doses of iNO provoke diffusion of the lipophilic NO through the lung tissue reaching nonventilated areas (“shunt areas”) and leading to pulmonary vasodilation with further reduction of pulmonary resistance but reversing the beneficial effect on oxygenation. 2) The kinetic theory is based on the pharmacokinetic rule that the time to total metabolism of a substance depends on the primary concentration. Pulmonary vasculature system is strictly dichotomous: in particular, from the pulmonary artery up to the final capillaries each vessel divides in two smaller ones without transverse connections and after the capillary system two vessels always rejoin to a larger one, until the pulmonary veins are reached. Thus vessels of shunt areas and of areas with ideal ventilation-to-perfusion ratio are finally united in the pulmonary venous system. Low doses of iNO diffuse into the intravascular space resulting in a low local concentration and acting on local vascular smooth muscle. Low concentration of NO is inactivated by hemoglobin before the venous vessel rejoins a shunt vessel, thus inducing vasodilation only in the ventilated area. Conversely, high doses of iNO correspond to high intracapillary concentrations. Thus the complete inactivation of NO by binding to hemoglobin requires more time resulting in decreased “afterload” for both ventilated and nonventilated areas, since NO remains partially active after rejoicing of the vessels.

The degree of lung ination may also be an important determinant of the effects of iNO. It has been reported that the recruitment of lung units by application of 10 cmH₂O continuous positive airway pressure (CPAP) augmented the improvement of oxygenation caused by inhaling 40 ppm NO in anesthetized dogs with oleic acid-induced lung injury (353). Application of CPAP reduced shunting regions from 48% of cardiac output to 21% and iNO at 40 ppm selectively reduced pulmonary artery pressure from 30 to 24 mmHg.

The advantage of iNO therapy is the pulmonary selectivity due to the inactivation of NO by its rapid combination with hemoglobin within the pulmonary circulation (373). The disadvantage of this therapy is the short duration of action, since many patients with chronic pulmonary hypertension or severe ARDS require continuous vasodilator therapy. Of note, the toxicity of prolonged NO exposure in humans with acute lung injury is unclear. Because NO is rapidly oxidized to NO₂ in the presence of high oxygen concentrations, the toxic effects of NO₂ may be of concern, especially during prolonged NO inhalation exposures. The effects of the cGMP-specific phosphodiesterase inhibitor Zaprinast on the pulmonary vasodilating effects of iNO in awake, spontaneously breathing lambs with pharmacologically induced pulmonary hypertension (188) have been investigated. The duration of the vasodilator response to iNO was markedly increased by Zaprinast infusion at all the three iNO concentrations. In particular with Zaprinast cotreatment, vasodilation induced by iNO was maintained for 88 min with only 4-min periods of iNO (40 ppm). Finally, the augmentation of the vasodilating effects of iNO by Zaprinast is temporally associated with increased net cGMP release from the pulmonary circulation.

Pulmonary hypertension is a frequent complication of severe COPD and a major cause of morbidity and mortality in this condition (192). Mean pulmonary artery pressure in patients with COPD is usually mild at rest but can rise to abnormally high levels on exercise. Although long-term oxygen therapy improves survival in hypoxic patients with COPD, it has a negligible effect on pulmonary hemodynamics. Several reports showed that the use of iNO in COPD patients may worsen ventilation/perfusion ratio (V/Q ratio) relationships and exacerbate systemic hypoxemia while lowering pulmonary vascular resistance (135, 217). When NO is delivered to well-ventilated alveolar units with fast time constants, the deleterious impact on gas exchange is avoided (377). Recently, it has been shown that long-term use of pulsed NO with oxygen (where spikes of NO are added at the beginning of inspiration) leads to sustained improvement in pulmonary hemodynamics without worsening hypoxemia in stable COPD patients (436). Benefits of the pulsed method include the reduced formation of nitrogen dioxide and met-hemoglobinemia. Further studies could shed light whether pulsed NO/oxygen treatment will lead to an improvement in exercise tolerance and survival in patients with hypoxemic COPD.

Finally, in neonates with persistent pulmonary hypertension, low-dose inhaled NO therapy has been shown to lead to a favorable long-term (1 yr) outcome with regard to need of extracorporeal membrane oxygenation without increased incidence of adverse effects (60). As an alternative for iNO, the efficacy has been assessed of inhaled O-nitrosoethanol gas (ENO) as a novel alternative means of providing NO bioactivity in the treatment of persistent pulmonary hypertension of newborns. ENO produced sustained improvements in postductal arterial oxygenation and systemic hemodynamics. Increases in methemoglobinemia were modest and toxic NO(x) were not detected. Thus ENO can improve oxygenation and systemic hemodynamics in neonates and seems to reduce...
rebound hypoxemia and production of toxic by-products (306).

Obviously, the other alternative of inhaled NO is the administration of NO donors (285), such as S-nitroso-N-acetylpenicillamine or sodium nitroprusside. An interesting development in this area is addition of NO-releasing capacity to well-known drugs, by the ester linkage of an NO-releasing moiety to the conventional drug molecule (221). In this way, various NO-donating drugs, such as NO-prednisolone and NO-releasing nonsteroidal anti-inflammatory drugs (NO-paracetamol, NO-aspirin, salbutamol-nitrate, etc.) are currently under investigation. Presently, these developments are still largely taking place outside the respiratory field.

VII. CONCLUSIONS AND FUTURE PERSPECTIVES

During the past 20 years we have witnessed an unforeseen revolution in airway physiology. The discovery of the delicate role of endogenous NO in the homeostasis of various cellular functions and the dynamic behavior of the airways has led to a new, rapidly progressing area of physiological science, which has direct bearing on our understanding of multiple airway diseases. However, we seem to be halfway only. The complexity of NO synthesis and the wide functional profile of its various bioactive forms have not been resolved in full detail yet. Ongoing research in this area will undoubtedly provide novel targets for subtle interventions in the prevention and treatment of airway disease.

Endogenous NO is synthesized by various, independently controlled enzymatic pathways. These can be constitutively expressed as well as induced and regulated at the gene-transcriptional level by several cytokines, chemokines, and mediators. Therefore, NOS is dynamically expressed, in both airway resident cells and infiltrating cells.

The bioactivity of NO is largely provided by S-nitrosothiols. However, NO can also be regarded as a free radical that interacts with reactive oxygen species, to form reactive nitrogen species. These include extremely bioactive products such as nitrite, nitrate, nitrotyrosine, and peroxynitrite.

NO has a definite role in gene expression during the embryological development of the airways and lung parenchyma. Based on its various bioactive forms and depending on a wide local concentration range, NO can have either protective or deleterious activities during states of airway damage, inflammation, and repair.

The potentially protective effects of NO include neuromodulation by mediating inhibitory noncholinergic nonadrenergic nerve activity, smooth muscle relaxation, attenuating airway hyperresponsiveness to bronchoconstrictor stimuli, downregulating Th1 cells and their proinflammatory activity, and the killing of invading microorganisms.

The potentially deleterious effects of NO (and reactive nitrogen species) include pro-inflammatory activities, such as vasodilatation and plasma extravasation of the bronchial circulation; increased airway secretions; impaired ciliary motility; promoting Th2 cell-mediated, eosinophilic inflammation; and necrosis and apoptosis (which may also be protective!).

NO is likely to be relevant in the pathogenesis of airway diseases, such as asthma, cystic fibrosis, and COPD. This can either be driven by polymorphisms in NOS genes, or by alterations in NOS gene expression caused by environmental exposure to allergens, cigarette smoke, or respiratory virus infections. The latter exposures appear to result in impaired endogenous protective activity by NO within the airways.

What can we expect during the coming years? It is not surprising that this powerful molecule is a target for drug development. This is supported by the fundamental concept that it seems to be preferable to restore physiological, endogenous inhibitory systems rather than developing unphysiological disease-combating strategies. The success of (inhaled) steroids, as the most effective anti-inflammatory agent in airway diseases, strengthens this view. Needless to say that steroids themselves are strong modulators of NO synthesis, by inhibiting inducible NOS and renewing constitutive NOS activity. What can be expected from the scientists in this field?

Cell biologists will further elucidate the complex synthesis and molecular pathways of NO metabolism, to find the major bioactive compounds and the right targets for intervention.

Geneticists will continue their search for (single nucleotide) polymorphisms in promoter regions and genes of NOS that might be associated with clinical phenotypes of airway disease. This should be expanded by genomic and proteomic approaches using microarray technology, to examine the expression of those genes in individual patients. At present, the development of gene transfer therapy seems to become a realistic approach in the treatment of, i.e., pulmonary hypertension. Recombinant adenovirus overexpressing eNOS (56, 201) or iNOS (44) has been shown to reduce pulmonary vascular resistance and remodeling in animal models of pulmonary hypertension. This approach should also be considered for intervention in other diseases with NO-driven pathophysiology.

Pharmacologists are having novel opportunities to modulate NO synthesis aimed to restore the balance between the protective and deleterious effects of NO. This is potentially beneficial in both airway (29) and alveolar diseases (212). Such interventions might be targeted in various ways, e.g., by using selective iNOS inhibitors (163,
427, 463), NO donors (221), or the above-mentioned usage of NOS gene transfer. In addition, pharmacologists should also explore the potential of arginase inhibitors (e.g., nor-NOHA), to reverse the increase in arginase activity and thereby the attenuation of protective NO activity during allergic inflammation (289, 469). Interestingly, these interventions might be fine-tuned by monitoring NO in exhaled air (276). Obviously, potential adverse effects, such as compromised host defense and pulmonary hypertension in the case of NO synthesis inhibition, should be carefully monitored.

Pathologists should examine the role of NO in modulating airway structure (airway remodeling) in chronic disease states. The antiproliferative effects of NO on airway smooth muscle are very promising in this respect.

Physiologists will further explore the functional role of endogenous NO in regulating airway patency. It cannot be excluded that NO is a major mediator in providing the most potent physiological protection against airway narrowing in healthy human subjects, namely, the bronchodilatation and bronchoprotection after a deep inspiration (119).

Clinicians should further expand their efforts in using exhaled NO as a marker of lung diseases (13). Monitoring adequate fractions of exhaled NO may not only be relevant for airway diseases, but also for parenchymal disease (425).

Hence, NO has already made it from the bench to the bedside, and it is not unlikely that new developments in this area will drastically change respiratory medicine during the coming 5–10 years.

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