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Forum Review

Nitric Oxide and Redox Signaling in Allergic Airway Inflammation

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ABSTRACT

A number of diseases of the respiratory tract, as exemplified in this review by asthma, are associated with increased amounts of nitric oxide (NO) in the expired breath. Asthma is furthermore characterized by increased production of reactive oxygen species that scavenge NO to form more reactive nitrogen species as demonstrated by the enhanced presence of nitrated proteins in the lungs of these patients. This increased oxidative metabolism leaves less bioavailable NO and coincides with lower amounts of *S*-nitrosothiols. In this review, we speculate on mechanisms responsible for the increased amounts of NO in inflammatory airway disease and discuss the apparent paradox of higher levels of NO as opposed to decreased amounts of *S*-nitrosothiols. We will furthermore give an overview of the regulation of NO production and biochemical events by which NO transduces signals into cellular responses, with a particular focus on modulation of inflammation by NO. Lastly, difficulties in studying NO signaling and possible therapeutic uses for NO will be highlighted. *Antioxid. Redox Signal.* 7, 129–143.

NITRIC OXIDE

(NO) levels in exhaled breath of patients with asthma

INFLAMMATORY DISEASES OF THE RESPIRATORY TRACT, such as asthma, bronchiectasis, and acute lung injury, are commonly associated with enhanced local production of NO. It has been presumed that the induction of type 2 NO synthase (NOS), the high-output form of NOS, is responsible for the increased levels of NO and its oxidation products in the expired breath (44, 84). Studies using selective NOS2 inhibitors found exhaled NO levels to decrease up to 95% in asthmatics, suggesting that the majority of exhaled NO is derived from NOS2 (57, 163). As the selectivity of pharmacological inhibitors is variable, it remains to be determined if the induction of NOS2 in the airway epithelium and inflammatory cells is the only source of the increased amount of NO in expired breath as seen in asthma. Recently, this notion has also been brought to question based on observations that exhaled NO levels remain increased in mice lacking NOS1 or 2, suggesting that NOS isoforms are redundant (18, 149). Other possible explanations for the elevated levels of NO in the expired air are the breakdown of *S*-nitrosothiols by *S*-nitrosoglutathione (GSNO) reductase, an enzyme shown to be more active in an ovalbumin-sensitization model of allergic airway disease (35), or the protonation of nitrite under acidic conditions, to form nitrous acid, which can decompose to release NO in the airways (108, 170). The pH of exhaled breath condensate of patients with acute asthma has been found to be as low as 5.2 (65), indicating that the lung surface of asthmatics is probably sufficiently acidic to cause formation of NO by this latter mechanism.

Measuring exhaled NO has been proposed as a noninvasive method to diagnose and monitor asthma as the levels closely correlate with markers of the disease, specifically eosinophilic inflammation and airflow parameters (74, 83). A recent study investigating the validity of exhaled NO for the diagnosis of asthma, found a 90% specificity in diagnosing asthma using exhaled NO levels, but a cutoff value for NO needs to be implemented in order for NO to be discriminatory for this disease (29).

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NO has multiple functions in normal lung tissue; it participates in regulating airway and vascular tone, mucin secretion, and influences mucociliary clearance. NO is also known for its bactericidal actions and provides additional host defenses against invading pathogens (34, 121), supported by studies demonstrating that NOS2-deficient mice are more susceptible to infection (121). Furthermore, NO may confer an early physiological defense against injury and inflammation by modulating the progression of the inflammatory process by affecting leukocyte adhesion and migration into lung tissue (60, 103) and by inhibiting phagocyte oxidase activation (16). In addition, NO has been shown to alter proinflammatory cytokine production by affecting cell signaling pathways and transcription factor activation and thereby altering gene expression (66, 132, 150), as will be further discussed later on.

NO DERIVATIVES IN ASTHMA

S-nitrosothiols have been measured in exhaled breath condensates, tracheal aspirates, and bronchoalveolar lavage fluids and normal values obtained from these studies vary markedly [from 80 n*M* to 1 μ*M* (20, 45, 51)]. This large variability could be due to the different ways of sampling, but it is also a consequence of the different specificities of the methods used to detect and quantify *S*-nitrosothiols. It is therefore hard to estimate the actual concentration of *S*-nitrosothiols in the epithelial lining fluid of the lungs, which will consist mainly of GSNO. However, intriguingly, most studies report levels of *S*-nitrosothiols in the above-mentioned samples from patients with asthma or cystic fibrosis (CF) to be markedly lower, or often undetectable, when compared with similar specimens from healthy subjects, despite normal or even enhanced airway NO production (45). These subnormal *S*-nitrosothiol levels could result from alterations in oxidative NO metabolism that would limit *S*-nitrosylation reactions, or accelerated oxidative or enzymatic degradation of *S*-nitrosothiols during inflammation. Indeed, oxidative metabolism has been presented by elevated protein tyrosine nitration in these conditions (77), which is largely a result of increased activation of the granulocyte peroxidases myeloperoxidase (MPO) and eosinophil peroxidase (EPO) (35). As mentioned earlier, GSNO reductase activity was found to be increased in an ovalbumin-sensitization model of asthma in guinea pigs. It was proposed that this GSNO reductase activity is leaking into the airway lining fluid from damaged airway epithelium (35). It is conceivable that decreased levels of *S*-nitrosothiols could contribute to asthma pathophysiology by minimizing the beneficial effects of *S*-nitrosothiols on airway smooth muscle tone and the anti-inflammatory properties of these NO derivatives.

The increased presence of nitrotyrosines, on the other hand, may also have deleterious effects with regard to airway hyperresponsiveness and surfactant function (22). Overall, the decreased levels of *S*-nitrosothiols, accompanied by increased levels of nitrotyrosine in asthmatics, strongly indicate altered or enhanced NO metabolism within the airways of these patients, which may have an impact on the overall pro- or antiinflammatory properties of NO.

REGULATION OF NO PRODUCTION

NO is synthesized by three isoforms of NOS in a reaction that converts L-arginine to L-citrulline and NO, using oxygen and NADPH as cofactors. NOS3 (eNOS) was originally identified in endothelial cells and NOS1 (nNOS) in neuronal cells. A mitochondrial variant of NOS has recently been shown to be widely distributed among tissues (78), but there is no consensus as to whether this is a variant of one of the known constitutive NOS isozymes or whether it is an entirely different protein (95). NOS1 and 3 are both dependent on increases in intracellular calcium to bind calmodulin, in order to be active and produce low levels of NO. NOS2 (iNOS) is expressed by a variety of cells and is responsible for high-output production of NO, independent of elevations in intracellular calcium, due to the tight binding of calmodulin even at low levels of calcium. In contrast to NOS1 and 3, NOS2 is regulated at the level of transcription and its expression is induced by immunologic and inflammatory stimuli, including interferon-γ, tumor necrosis factor-α (TNFα), and lipopolysaccharide (LPS).

NOS enzymes are also regulated at the level of translation (100), posttranslation, and degradation. Phosphorylation of NOS enzymes by Akt (25), calcium/calmodulin-dependent protein kinases (91) and members of the AMP-activated kinases PKA and G (14, 15) has been reported to both increase and decrease NOS activity, depending on the residue that is targeted by phosphorylation. In addition, NOS activity can be influenced by regulation of its proteolytic degradation. Following inhibition of the interaction between one of the chaperones and NOS or in response to treatment with guanidine compounds, NOS proteins are ubiquitinated and degraded via the proteasomal pathway (8, 90). Conditions that are accompanied by suboptimal amounts of the substrate for NOS, L-arginine, the essential cofactor tetrahydrobiopterin, or an altered composition of the NOS membrane complex can promote the generation of superoxide ($O_2^{\bullet -}$) by all isoforms (23, 131, 157, 159). This cogeneration of NO and $O_2^{\bullet -}$ by NOS can lead to the formation of more reactive nitrogen species (RNS), like peroxynitrite ($ONOO^-$), and lesser availability of NO *per se*. This could be relevant to allergic airway disease, because cationic proteins like the eosinophil product major basic protein (MBP) can limit substrate availability for NOS, by inhibiting the cellular uptake of L-arginine (55).

NO SIGNALING IN THE LUNG

The traditional view on signal transduction encompassed an image of free diffusing molecules within the cell, and NO, along this line of thinking, has ideal properties as a signaling molecule, because it diffuses rapidly through aqueous phase and membranes. In recent years, however, this notion has been replaced with the appreciation that signaling takes place within confined subcellular compartments to insure specific targeting and propagation of signals (21). For NO, this implies that the source of NO and the target molecules should be colocalized. The three different isoforms of NOS enzymes and their multiple splice variants are indeed discretely localized within subcellular compartments and form complexes with different

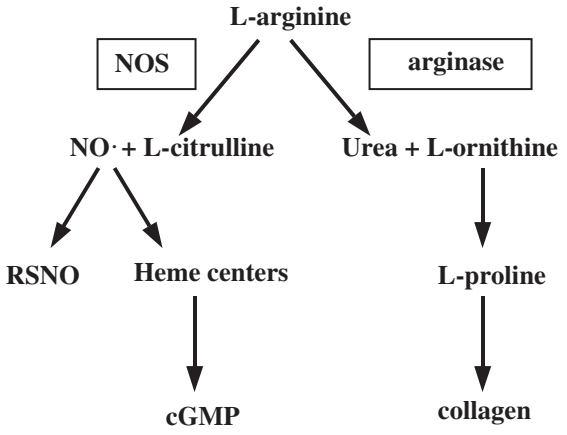

FIG. 1. Schematic representation of L-arginine consuming pathways. L-Arginine can be converted by NOS into NO and L-citrulline. NO can subsequently react with sulfhydryl groups of cysteine residues in proteins in an *S*-nitrosylation reaction or NO can react with heme centers and modify protein function. Alternatively, L-arginine can be converted by arginase to form urea and L-ornithine, the latter a precursor for L-proline and collagen synthesis.

subsets of proteins through the interaction with scaffolding proteins, to enable specific targeting of proteins with distinct functions. All three isoforms are known to reside in caveolae, the microdomains of the plasmalemma that have been implicated in transcytosis and signal transduction events (126). NOS enzymes have been shown to interact directly with caveolins, the integral membrane proteins of caveolae under basal conditions, and their activity requires dissociation from caveolin (42, 46, 49, 92). This tight regulation of localization, production, and activity makes NOS ideal for generating NO as a signaling molecule that can regulate physiological processes.

L-arginine availability is another regulator for the production of NO and is in part determined by its uptake by the cationic amino acid transporter 1 (CAT1). CAT1 is a member of the NOS membrane complex, ensuring direct delivery of L-arginine to NOS and optimizing NO release (115). In this regard, as mentioned earlier, cationic proteins like the eosinophil product MBP can inhibit the cellular uptake of L-arginine and consequently inhibit NO production by alveolar macrophages and airway epithelial cells (55).

The availability of L-arginine, in addition to being regulated by uptake, is also determined by its consumption in an alternative reaction in which L-arginine is converted by arginase to form urea and L-ornithine, the latter being an essential precursor for the synthesis of polyamines and L-proline (144) (Fig. 1). Importantly, a recent study demonstrated no change in NOS2 expression, but found both arginase isoforms 1 and 2 to be up-regulated in animal models of allergic airway disease, as well as in patients with asthma (169). The induction of arginase could be due to Th2 cytokines, like interleukin (IL)-4 and IL-10, that are a characteristic feature of asthma (98, 120). This new finding is intriguing, as it implies lower availability of substrate for NOS and increased substrate for collagen formation in the form of L-proline in allergic airways disease. In this regard, it is of interest to note that in a bleomycin-induced model of fibrosis of mouse lung, both arginase isoforms were found to be up-regulated (31).

METABOLISM OF NO TO RNS

Oxidation of NO to RNS

A number of proinflammatory mediators that are present in asthmatic airways can cause an increase in the production of NO, as well as reactive oxygen species. In conditions where NO and $O_2^{\bullet -}$ are produced at the same time and in close proximity, such as in inflammatory cells like eosinophils and neutrophils, which are known to infiltrate the lungs of asthmatics, the typical nitrating species, nitrogen dioxide ($NO_2^{\bullet}$) and $ONOO^-$, that can modify tyrosine residues in proteins are formed (68). Both eosinophils and neutrophils contain the enzyme NADPH oxidase, which converts O_2 into $O_2^{\bullet -}$, which in turn rapidly reacts with NO to form $ONOO^-$. Alternatively, the leukocyte peroxidases EPO and MPO, which are stored in granulae and released upon activation, catalyze the formation of $NO_2^{\bullet}$ from nitrite (NO_2^-) and hydrogen peroxide (H_2O_2) (154, 158) (Fig. 2). Studies using MPO and EPO knockout mice have confirmed the importance of these enzymes in the formation of nitrotyrosines *in vivo* (27, 67).

In addition to the formation of RNS by inflammatory cells, nonphagocytic cells are also a source of these oxidants. For instance, mitochondrial respiration, xanthine/xanthine oxidase,

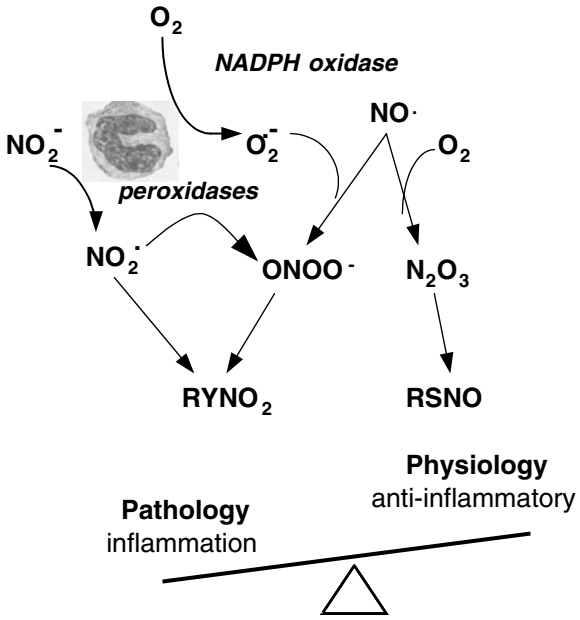

FIG. 2. Oxidative metabolism of NO and the formation of RNS. Oxidation of NO is affected by the redox status of the cell, the presence of hydrophobic regions, and other reactants, such as oxygen and transition metals. Furthermore, activation of oxidases and peroxidases can decrease NO by causing the production of highly reactive RNS, like $ONOO^-$ and $NO_2^{\bullet}$.

and nonphagocytic oxidases, represent important sources of $O_2^{\bullet -}$ (96), and as mentioned earlier, NOS isoforms are expressed in a variety of cells. Collectively, the biochemical pathways that are required for the formation of RNS are present in both inflammatory and noninflammatory settings (40). For a review on oxidative events in asthma, see Andreadis *et al.* (4).

Tyrosine nitration

Nitrotyrosine formation has been considered to be a footprint for the presence of $ONOO^-$ or $NO_2^{\bullet}$. Increased nitrotyrosine reactivity has been detected in a variety of diseases of the respiratory tract, such as asthma, acute lung injury, CF, chronic obstructive pulmonary disease (COPD), and acute respiratory distress syndrome (77, 118, 141, 168).

Although speculations exist that nitration of tyrosines impairs the ability of kinases to phosphorylate the same residues (106) or, on the other hand, that nitrotyrosines could mimic the tyrosine phosphorylation status (9), experimental evidence for these outcomes in diseases of the airways is lacking. Other data suggest that nitrated proteins are more susceptible to degradation by the proteasome (53, 54, 143), implying that removal of nitrated proteins may serve as a protective mechanism against nitrative stress. Evidence indicates that nitration is reversible and that nitrated proteins can be repaired by a so-called denitrase activity (22, 50). This activity was induced by LPS, destroyed by heat and trypsin, and importantly appeared to be substrate-dependent. However, the denitrase enzyme remains to be further purified and characterized in order to substantiate its relevance.

One of the proteins that has been shown to be nitrated and functionally impaired by the modification is IL-8, one of the chemokines for neutrophils. This cell type has been implicated in severe asthma (89) and in COPD. Studies have shown that the addition of $ONOO^-$ to IL-8 inhibits its binding to neutrophils and subsequently reduces its chemotactic potential *in vitro* (136). On the other hand, the antiinflammatory effects of the cytokine IL-10 *in vitro* are enhanced by $ONOO^-$ treatment (123). Furthermore, nitration of cytoskeletal proteins like actin and α-tubulin contributes to alterations in cell morphology and microtubular organization and causes intracellular redistribution of motor proteins (30). In the context of the lung, $ONOO^-$ has been shown to cause increased hyperresponsiveness to methacholine and to inhibit surfactant function through nitration of surfactant protein A (135). Although these findings are suggestive of an important role of tyrosine nitration of key target proteins in allergic airway diseases, a causal role for these events *in vivo* remains largely to be determined.

S-NITROSATION / *S*-NITROSYLATION

Many of the biological actions of NO are mediated through the reaction with iron or heme-containing proteins such as guanylyl cyclase, cytochrome *c* oxidase (152), lipoxygenase (124), prostaglandin H synthase (125), and MPO (1, 7, 48). The binding of NO to heme iron of soluble guanylyl cyclase, causing its activation and subsequent rise in cyclic GMP (cGMP) production, was the first evidence that NO can act as a signaling molecule (61). cGMP acts as a second messenger, activating cGMP-dependent protein kinase, cGMP-gated cation channels, and cGMP-regulated phosphodiesterase. Some of the effects of NO that are mediated in this manner include smooth muscle relaxation, neurotransmission, and platelet aggregation and disaggregation.

Approximately a decade ago, redox-sensitive protein cysteine residues were determined to represent alternative targets for NO. They can undergo so-called *S*-nitrosylation, the attachment of NO to sulfhydryl groups in proteins [whereas the attachment of NO to nucleophilic centers in general is called *S*-nitrosation (140)]. This results in changes in the tertiary structure and, in many cases, functional alterations of the proteins involved (148). *S*-Nitrosylation has emerged as the prototypic redox-based posttranslational modification and has often been compared with phosphorylation (107). This analogy to other modes of signal transduction forms the main reason why the NO-dependent modification of sulfhydryl groups is called *S*-nitrosylation as opposed to *S*-nitrosation, which is the chemically correct term. The importance of this newly described posttranslational modification is emphasized by the large number of proteins from all major classes that have been characterized as potential targets for *S*-nitrosylation to date. These include channel/transporters, structural proteins, transport/storage proteins, metabolic enzymes, signaling proteins, and transcription factors (11) (Table 1).

S-Nitrosylation requires (enzymatic) activation of NO to an NO^+ equivalent product [N_2O_3 (64), iron NO^+ complexes, per-

Table 1. Potential Targets for *S*-Nitrosylation

Protein	*Effect*	*References*
NMDA receptor	Inhibition	Lipton *et al.*, *Nature* 364: 626–632, 1993.
p21 H-ras	Activation	Lander *et al.*, *J Biol Chem* 272: 4323–4326, 1997.
Ryanodine receptor	Activation	Xu *et al.*, *Science* 279: 234–237, 1998.
JNK	Inhibition	Park *et al.*, *Proc Natl Acad Sci U S A* 97: 14382–14387, 2000.
Caspase 3	Suppresses activity	Mannick *et al.*, *J Cell Biol* 154: 1111–1116, 2001.
p50	Inhibits DNA binding	Marshall *et al.*, *Biochemistry* 40: 1688–1693, 2001.
Thioredoxin	Required for activity	Haendeler *et al.*, *Nat Cell Biol* 4: 743–749, 2002.
MMP9	Activation	Gu *et al.*, *Science* 297: 1186–1190, 2002.
IKKβ	Inhibition	Reynaert *et al.*, *Proc Natl Acad Sci U S A* 101: 8945–8950, 2004.

NMDA, *N*-methyl-D-aspartate; MMP9, matrix metalloproteinase 9.

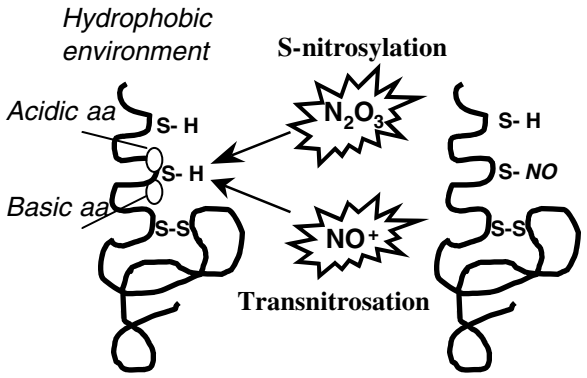

FIG. 3. S-nitrosylation of proteins. *S*-Nitrosylation of cysteine residues in proteins is believed to occur within an acid–base motif and mainly hydrophobic environment where NO rapidly reacts with oxygen to form the *S*-nitrosating species N_2O_3. NO can also be transferred from one thiol to another in a transnitrosation reaction.

haps $ONOO^-$] (155), because NO itself does not react with thiols directly. A nitroso functional group, that is, NO^+, can also be transferred from a nitrosothiol to another cysteine residue in a transnitrosation reaction (63) (Fig. 3). Intriguingly, not all cysteine residues are equally susceptible to *S*-nitrosylation. In fact, the majority of proteins that have been shown to be targeted by *S*-nitrosylation are only modified at a single critical cysteine residue. This cysteine is generally located within an acid–base and overall hydrophobic structural motif that may only be apparent from the tertiary or quaternary structure of the protein (147). The exact biochemical pathways involved in *S*-nitrosylation *in vivo* are still unknown, but appear to depend mainly on the microenvironment of the protein, which determines the pK_a of the cysteine being targeted (6) and the accessibility to nitrosating intermediates.

Conversely, *S*-nitrosylation of proteins is a transient modification, and can be reversed by several nonenzymatic pathways, such as reactions with other cellular thiols (11, 64, 75), ascorbate (80), various heme proteins (145), copper ions (167) and light (139). Furthermore, enzymatic pathways that break down *S*-nitrosothiols also have been described *in vitro* and *in vivo*. They include thioredoxin reductase (122), xanthine oxidase (153), Cu/Zn superoxide dismutase (76), and a recently identified cellular GSNO reductase, which utilizes NADH and glutathione (GSH) as cofactors (Table 2). It should be noted that the latter enzyme is otherwise known as GSH-dependent formaldehyde dehydrogenase or alcohol dehydrogenase class III (104). Further studies are required to characterize the role of this enzyme in the metabolism of *S*-nitrosothiols and to investigate if other dehydrogenases share this SNO reductase activity.

Overall, the various biochemical and enzymatic pathways that regulate *S*-nitrosylation and denitrosylation indicate a tight regulation of these events, and provide strong arguments for *S*-nitrosylation as an important biochemical posttranslational modification associated with NO. Furthermore, *S*-nitrosothiols can serve as a systemic reservoir of NO (45), tightly regulating the location and amount of NO that is necessary for the control of protein function and signal transduction.

TABLE 2. DEGRADATION OF *S*-NITROSOTHIOLS

Enzymatic
Alcohol dehydrogenase class III
Xanthine oxidase
Thioredoxin reductase
Cu/Zn superoxide dismutase
Nonenzymatic
Ascorbate
Light
Heme proteins
Low-molecular-weight thiol
Transition metal ions

INVESTIGATIONS INTO THE ROLE OF NO IN ASTHMA

Approaches to determine the involvement of NO in allergic airway disease have included pharmacological inhibition of one or more NOS isoforms and the use of mice containing targeted deletions of NOS isozymes. Because of the suggested involvement of NOS2, studies of airway inflammation have focused mostly on NOS2-knockout mice or NOS2 inhibitors and have suggested that NOS2-derived NO contributes to lung inflammation, lung injury and mortality in influenza virus pneumonitis (2), acute lung injury by LPS or ozone inhalation (81, 86, 127), as well as eosinophilic infiltration following ovalbumin immunization and challenge (24, 161). Nevertheless, several other reports have failed to demonstrate a contribution of NOS2 to allergic airway inflammation (24, 36), and in some studies of oxidant-induced lung injury, NOS2 deficiency actually resulted in increases in neutrophil influx (81, 88), which is more consistent with the known antiinflammatory properties of NO. Furthermore, whereas NOS2 inhibition or deficiency only seemed to affect inflammation and protein nitration, it appeared to have no effect on other features of allergic airways disease, such as airway hyperreactivity, airway cell proliferation, and eosinophilia (24, 33, 87), although conflicting reports exist (33, 67). However, recent pharmacological studies have suggested a role for the constitutive isoforms of NOS rather than NOS2 in eosinophilic inflammation in models of allergic airways inflammation (10), and the constitutive NOS isozymes have been associated with changes in responsiveness to methacholine challenge (37). For example, airway hyperreactivity in a mouse model of allergic airway disease was suppressed in animals deficient in NOS1, but increased in NOS3-deficient mice (24). These previous findings are also consistent with recent linkages of NOS1 and NOS3 gene polymorphisms with human asthma and elevated levels of exhaled NO (52, 156).

One of the main issues in interpreting results obtained from generic NOS knockout mice is that the three different isoforms may be redundant. For instance, mice lacking either NOS1 or 3 display increased exhaled NO levels (149). Additionally, NO affects immune functions and loss of NOS2 has been suggested to alter immunization *per se*, which compromises the

studies evaluating NO in allergic airways disease (82). Thus, improved mouse models that use conditional or tissue-specific targeting approaches will be required to elucidate the role of various NOS isoforms in allergic airway inflammation.

NO AND INFLAMMATION

NF-κB

NF-κB is a versatile transcription factor that plays a pivotal role in inflammation, cell survival, and proliferation. NF-κB can be induced by >150 different stimuli and participates in the transcription of >150 different genes. Proinflammatory mediators induced by NF-κB include adhesion molecules (intercellular adhesion molecule 1), enzymes (NOS2, cyclooxygenase-2), cytokines (TNFα, IL-1β), and chemokines (eotaxin, RANTES) (128).

NF-κB is maintained in a latent form in the cytoplasm via sequestration by IκB inhibitory proteins. Canonical NF-κB-activating stimuli cause the inducible degradation of IκB proteins, unmasking the nuclear localization signal of NF-κB, resulting in its nuclear translocation, binding to NF-κB motifs, and the activation of gene transcription (47, 79, 113). The enzyme complex responsible for phosphorylation of IκB on specific serine residues is inhibitory κB kinase (IKK), which consists of at least three subunits: IKKα, IKKβ, and IKKγ (the latter also known as NF-κB essential modulator) (165). Although IKKα and IKKβ are both catalytically active, studies in knockout mice have demonstrated that IKKβ is responsible for degradation of IκB in response to many signals (47, 79, 102, 114). In contrast, IKKα activation requires the NF-κB-inducing kinase (160), which is activated by a different subset of stimuli, such as CD40 ligand (19). IKKα plays an important role in the transcriptional activation of NF-κB responsive genes by inducing the processing of p100 to p52 (138) and by phosphorylation of histone H3 (5, 162). IKKγ is the regulatory subunit responsible for stabilizing the IKK complex and allowing interaction with upstream regulatory proteins (134).

NF-κB and asthma

As reviewed earlier, considerable evidence exists that NO plays a role in the inflammatory processes associated with allergic airway disease. One of the mechanisms by which NO can regulate inflammation is by affecting signaling pathways that culminate in the activation of NF-κB, a transcription factor that is believed to play a central role in the pathogenesis of asthma. Excessive activation of NF-κB has been shown to occur in alveolar macrophages obtained from induced sputum, as well as in airway epithelial cells obtained from bronchial brushings from asthmatics as compared to normal individuals (58). Furthermore, allergens that are associated with allergic asthma (130, 146) as well as viral infections and ozone that cause asthma exacerbations, are known to activate NF-κB *in vitro* (73, 112). Lastly, corticosteroids that are the most effective treatment for asthma exert their antiinflammatory effects in part by preventing the activation of NF-κB (56).

NO and NF-κB

Cellular studies have suggested several mechanisms by which NO regulates NF-κB (for review, see 72 (Fig. 4). First,

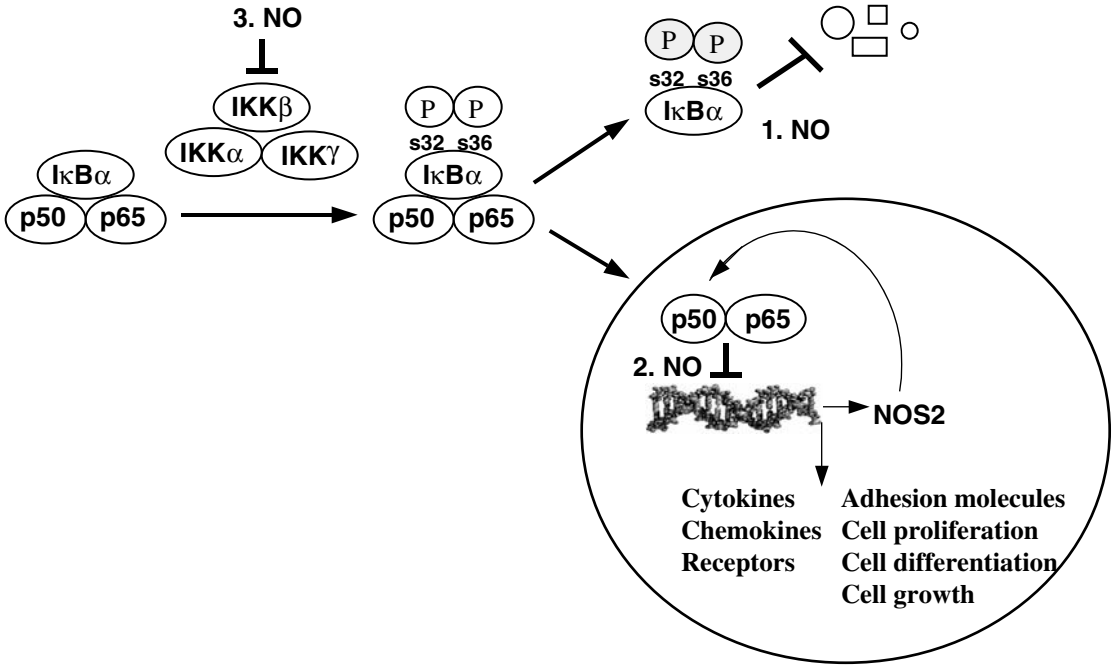

FIG. 4. Influence of NO on the NF-κB signaling pathway. NO has been shown to inhibit the proteasomal degradation of IκBα, increase transcription of IκBα, and stabilize the mRNA (**1**). DNA binding activity of NF-κB in addition, is also inhibited by *S*-nitrosylation of cysteine residue 62 within the p50 subunit (**2**). IKK is also susceptible to repression by NO (**3**). Collectively, a feedback mechanism appears to exist by which NF-κB–mediated induction of NOS2 and the consequent rise in NO production repress NF-κB.

it is believed that a feedback mechanism exists by which NF-κB-mediated induction of NOS2 produces NO, which in turn inhibits NF-κB and consequently suppresses the inflammatory response. Mechanisms by which NO represses NF-κB include the increased transcription of IκBα, the stabilization of the mRNA, and inhibition of the proteolytic degradation of IκBα (129). *S*-Nitrosylation of cysteine residue 62 within the p50 subunit of NF-κB has furthermore been shown to prevent DNA binding activity and subsequent inhibition of the expression of NF-κB related proteins (109, 111). Recent studies have also demonstrated that IKKβ is susceptible to inhibition by oxidants (93, Reynaert *et al., Proc Natl Acad Sci U S A* 101: 8945–8950, 2004). Marshall and Stamler furthermore have alluded to a target for NO-mediated repression of NF-κB upstream of the degradation of IκBα in Jurkat T-cells, which could represent IKK. In contrast, NO-mediated repression of NF-κB in lung epithelial cells occurred predominately at the level of DNA binding, and thus could reflect *S*-nitrosylation of p50 (110). These findings suggest that the mode of inhibition of NF-κB by NO may be cell type-dependent. The recent finding that stimulation of cells with TNFα, which activates IKK and NF-κB, reduces overall protein *S*-nitrosylation (62) is certainly consistent with an inhibitory effect of *S*-nitrosylation on IKK and NF-κB activation, although no specific targets for denitrosylation were identified. In addition to inhibitory effects of NO on NF-κB, several studies have also reported stimulatory effects of NO on NF-κB, potentially related to *S*-nitrosylation of upstream factors such as p21Ras (97). Collectively, ample evidence exists in support of a regulatory role of NO on NF-κB activation. It appears that this regulation occurs at various levels, in many cases through *S*-nitrosylation of selected cellular targets, and that the overall effects on NF-κB depend strongly on the cell type, the localization and extent of NOS induction, and/or activation of NF-κB (17). Whether alterations in *S*-nitrosylation are also involved in regulating NF-κB or other transcription factors in airway inflammation *in vivo* remains unclear. Overall effects of such NO-dependent regulation of NF-κB on inflammation may not be easy to predict, as NF-κB activation also regulates the production of antiinflammatory cytokines and apoptosis of granulocytes, thereby controlling later resolution stages of inflammation (41, 94, 99), in addition to its well known involvement in the initiation of inflammation.

NO and apoptosis

Asthma is commonly associated with impaired apoptosis of inflammatory cells in lung tissue, specifically eosinophils, leading to the accumulation of these cells and their potent mediators, aggravating the inflammatory state as opposed to restoring normal tissue homeostasis. The lack of clearance of eosinophils may be correlated with the severity of the disease (28), and it has been demonstrated that glucocorticoids exert their antiinflammatory effects in part by targeting inflammatory cells to undergo apoptosis (26). Asthmatic lung epithelium, on the other hand, is more susceptible to oxidant-induced apoptosis, and this can lead to sloughing of airway epithelial cells and consequently a loss of barrier and regulatory function (13).

An extensive number of reports have clearly demonstrated that NO and its derivatives are involved in regulating cell death. In the context of asthma, NO and *S*-nitrosothiols have been shown to inhibit eosinophil apoptosis *in vitro*. For instance, NO has been shown to disrupt Fas-induced apoptosis of eosinophils at the level of, or proximal to, activation of c-Jun N-terminal kinase (JNK) (59), and inhibition of endogenous NO production was found to increase the expression of Bcl-2 in eosinophils isolated from blood of asthmatics through effects on the mitogen activated protein kinases ERK (extracellular signal-regulated kinase) and p38, indicating that endogenous NO can serve to promote apoptosis via suppression of Bcl-2 expression (105).

S-Nitrosylation of caspases, a family of cysteine proteases that are responsible for many proteolytic events that occur during apoptosis, has received much attention as a NO-dependent inhibitory mechanism of cellular apoptosis. For instance, Fas-induced apoptosis not only requires the cleavage of caspase-3 zymogens into their active form, but is also associated with denitrosylation of the catalytic-site cysteine (32, Mannick *et al., Science* 284: 651–654, 1999). Ligation of Fas concurrently induces heme iron nitrosation of cytochrome *c* and its subsequent release from the mitochondria into the cytoplasm, where it participates in the formation of the apoptosome. It was proposed that Fas stimulation induces a subtle conformational change in cytochrome *c*, which would increase the reactivity of its heme iron to NO that is locally produced in the mitochondria (137). Caspases 9 and 8 have also been shown to be inhibited by *S*-nitrosylation directly (85, 151), and additional evidence exists that NO prevents the correct assembly of the apoptosome and subsequent processing of procaspases 9, 3, and 8 (166). In addition, endogenous NO was shown to control cell cycle and prevent apoptosis of pulmonary epithelial cells, as depletion of NO in these cells caused alterations in cell cycle and increased apoptosis in a cGMP-dependent fashion (71).

Proapoptotic effects of NO may be exerted by its local production in the mitochondria, where it has the potential to regulate respiration and affect mitochondrion-regulated apoptosis. For instance, NO can induce necrotic cell death by inhibiting mitochondrial respiration through the inhibition of cytochrome *c* oxidase, by binding to either the heme iron or the copper center and competing with oxygen (12). This will lead to a depletion of energy and consequently to necrosis. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by NO via *S*-nitrosylation of the active-site cysteine (116, 117) represses glycolysis, leading to a depletion of ATP production, and subsequently causes necrosis.

As an example of NO-induced apoptosis, both NO gas and GSNO have been shown to cause apoptosis of neutrophils, another cell type that has been suggested to play a role in severe asthma (89). The interaction of NO with $O_2^{\bullet -}$ and the formation of $ONOO^-$ were shown to be partially responsible for the induction of apoptosis, as the effects were mitigated by treatment with superoxide dismutase (38, 39), although the precise site of action of NO in the apoptotic cascade was not shown.

Collectively, these reports demonstrate that NO and its derivatives play a role in regulating cell death. NO-induced cell death, whether it is in the form of apoptosis or necrosis, may serve to kill invading pathogens and limit the extent of inflammation by enhancing the removal of inflammatory cells. It should be noted that necrotic cell death can actually enhance inflammation and NF-κB activation (101). The effects

of NO on cell death are complex and may be difficult to predict *in vivo*. In physiological conditions, the antiapoptotic effects of NO and *S*-nitrosothiols in particular may prevail, whereas superphysiological concentrations of NO may lead to death of host cells.

STUDIES OF NO FUNCTION *IN VIVO*

To determine the involvement of NO in the regulation of airway inflammation *in vivo* at the molecular level, and to identify the proteins that are regulated by NO, improved techniques will be required to demonstrate the involvement of *S*-nitrosylation in disease states and identify and localize specific *S*-nitrosylated proteins. The majority of proposed targets for *S*-nitrosylation have been investigated *in vitro*. In fact, rarely has an endogenous source of NO been directly linked to this posttranslational event. A major difficulty in assessing the role of endogenous protein *S*-nitrosylation has been the reliable detection of this modification within proteins *in vivo* and a lack of quantitative information regarding *S*-nitrosylation, which is due mainly to the unstable nature of *S*-nitrosothiols in the presence of metal ions. In addition, antibodies developed against the *S*-nitrosocysteine moiety have been used with variable success due to the small size of the epitope and its well known instability. The most widely used and accepted technique to date to demonstrate *S*-nitrosylation of proteins is chemiluminescence, coupled to either chemical reduction or photolysis of the S-NO bond. Recently developed strategies to selectively derivatize and isolate *S*-nitrosothiols from cell lysates and tissue homogenates (69, 70) (Fig. 5) will allow a more systematic approach to identify major cellular targets for protein *S*-nitrosylation. However, great care needs to be taken to avoid false positive and negative outcomes, including the complete blocking of all reduced sufhydryl groups and the presence of metal chelators to prevent transnitrosation or denitrosylation reactions. Nonetheless, this approach may aid in elucidating the role of *S*-nitrosylated proteins in inflammation in general, and in the pathology of airway diseases such as asthma specifically.

The identification of nitrated proteins may be less challenging as the nitrotyrosine moiety is a chemically more stable event, which makes the production of antibodies directed against nitrotyrosine an easier process, although specificity could still be problematic. A wide variety of target proteins have been identified *in vitro*, as well as *in vivo*. Establishing tyrosine nitration as an important posttranslational event, linking the modification directly to a change in protein function *in vivo*, however, remains an important challenge in this aspect of NO research.

CAN NO BE USED AS A THERAPEUTIC?

The inhalation of NO is already being used in the treatment of respiratory diseases, such as pulmonary hypertension of newborns, but concerns exist about its toxicity (133). For lung diseases such as asthma that appear to be associated with impaired NO functions, which may affect the regulation of airway tone and mucociliary clearance, a rationale for NO inhalation therapy would be apparent, although administered NO will most likely undergo the same fate as endogenously produced NO. As many of the beneficial actions of NO are attributed to *S*-nitrosothiols, these compounds could represent a more appropriate therapy. In this regard, GSNO represents an endogenous bronchodilator that is two log orders more potent than theophylline (43). Furthermore, in CF patients, aerosolized GSNO acutely increased oxygen saturation, and CF cells treated with GSNO *in vitro* showed enhanced maturation of the CF transmembrane regulator and restoration of normal functionality (3, 142, 164). In addition, the *S*-nitrosylating gas, *O*-nitrosoethanol, which does not react with oxygen, improved ventilation to perfusion matching in an animal model of pulmonary hypertension (119). Whether *S*-nitrosothiols could improve asthma by relaxing airway smooth muscles and

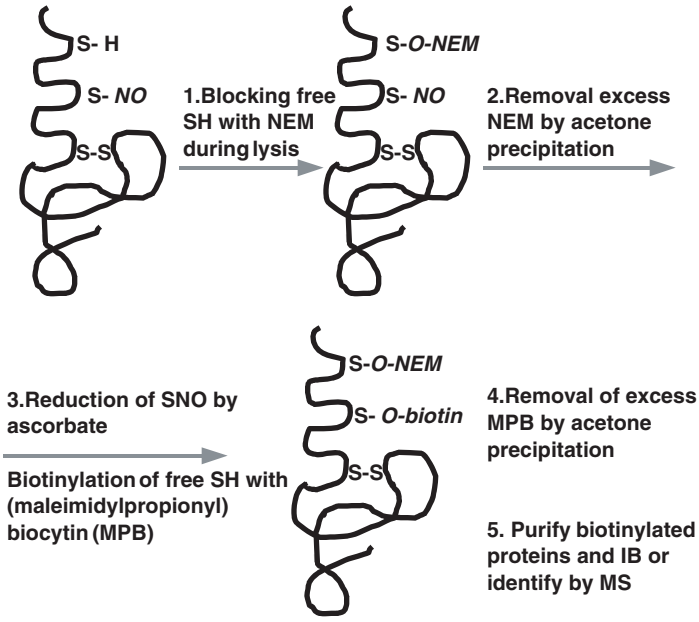

FIG. 5. Principle of biotin derivatization to identify *S*-nitrosylated proteins. In the first step, free sulfhydryl groups are blocked, after which the S-NO bond is reduced by ascorbate, and newly formed sulfhydryls are finally labeled with biotin. Biotinylated proteins can subsequently be purified on streptavidin columns and identified by western blotting for a specific protein or by mass spectrometry. NEM, *N*-ethylmaleimide. Adapted from Wu *et al.* (*J Biol Chem* 273: 18898–18905, 1998) and Jaffrey and Snyder (69).

act to repress airway inflammation remains to be determined, because indications exist that the enzyme that metabolizes GSNO is elevated in allergic airway disease (35), potentially limiting the beneficial effects of this strategy.

FUTURE DIRECTIONS TO STUDY NO SIGNALING IN THE LUNG

Compelling evidence exists that a number of respiratory diseases have features of altered NO metabolism that may be expected to affect its biological properties, as has been reviewed here. Over the years, NO has firmly been established as a potent second messenger molecule, influencing a myriad of physiological and pathophysiological processes, through cGMP-dependent pathways and *S*-nitrosylation reactions. Linking the alterations in NO metabolism observed in different disease states to altered posttranslational modifications of proteins by NO is the main challenge that lies ahead in this research field. Due to the unstable nature of *S*-nitrosylation, identifying targets in an *in vivo* setting represents a major difficulty that will need to be overcome. Derivatization techniques and advances in mass spectrometry should help to accomplish this goal. An improved understanding of the biochemical pathways that dictate NO availability will also be required in order to optimize the beneficial functions of NO, while limiting the formation of more damaging RNS. Lastly, an improvement of the available mouse models will be required to overcome the problems and controversies obtained from the use of generic knockout mice. This includes models that would overexpress or be deficient in NOS in selective compartments of the lung, like airway epithelium, smooth muscle, vessels, and inflammatory cells, to dissect out the relative contributions of NO generated therein. Given the rapid technical advancements in these areas, it is likely that better insights into the role of NO in pulmonary diseases will be forthcoming.

NOTE ADDED IN PROOF

Since the submission of our original paper, work by our laboratory was published showing that IKKβ is a direct target for *S*-nitrosylation (Reynaert *et al., Proc Natl Acad Sci U S A* 101: 8945–8950, 2004).

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ABBREVIATIONS

CAT1, cationic amino acid transporter 1; CF, cystic fibrosis; cGMP, cyclic GMP; COPD, chronic obstructive pulmonary disease; EPO, eosinophil peroxidase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; IKK, inhibitory κB kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MBP, major basic protein; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; $NO_2^{\bullet}$, nitrogen dioxide; NOS, nitric oxide synthase; $O_2^{\bullet -}$, superoxide; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; TNFα, tumor necrosis factor-α.

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