Nitric Oxide, Nitrotyrosine, and Nitric Oxide Modulators in Asthma and Chronic Obstructive Pulmonary Disease

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Current Allergy and Asthma Reports 2003, **3:**121–129
Current Science Inc. ISSN 1529-7322
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Nitric oxide (NO), a simple free-radical gas, elicits a diverse range of physiologic and pathophysiologic effects, and plays an important role in pulmonary diseases. Nitrosative stress and nitration of proteins in airway epithelium may be responsible for steroid resistance in asthma and their ineffectiveness in chronic obstructive pulmonary disease (COPD), supporting the potential role of future therapeutic strategies aimed at regulating NO synthesis in asthma and COPD. In this article, we review the potential role of NO modulators (NO synthase inhibitors and NO donors), which, if given on a regular basis, may have clinical benefit in asthma and COPD.

Introduction

Nitric oxide (NO) plays an important role in pulmonary diseases and the high intensity of nitrosative stress significantly contributes to the pathogenesis and clinical outcome of asthma and chronic obstructive pulmonary disease (COPD) [1••].

Glucocorticoids are potent anti-inflammatory agents and are widely used in the treatment of asthma. Treatment with inhaled corticosteroids reduces inducible NO synthase (iNOS) messenger RNA (mRNA) expression [2] and exhaled NO [3] in asthmatic patients by reducing inflammation in asthmatic airways. Some patients, however, with severe or glucocorticoid-dependent asthma, require long-term systemic glucocorticoids in addition to their current treatment to control the disease. These patients present an ongoing inflammation of the airways, usually characterized by an increased number of neutrophils and activated T lymphocytes [4].

The response to inhaled corticosteroids (ICS) in COPD is different from that in asthma because the predominant inflammatory cell infiltrates in both these conditions are distinct [5]. Several large clinical trials have shown no beneficial effect of ICS on the decline of lung function over 3 years in COPD [6]. ICS and oral steroids have no effect on inflammatory cells, cytokines, or proteinases in COPD [5].

Given the relative ineffectiveness of corticosteroid treatment in COPD, and the risk of adverse effects, more specific therapies directed against the reduction of inflammation are desirable. We speculate that nitrosative stress and nitration of proteins in airway epithelium may be responsible for steroid resistance in severe (steroid-dependent and steroid-resistant) asthma and their ineffectiveness in COPD, supporting the potential role of future therapeutic strategies aimed at regulating NO synthesis in asthma and COPD.

It is likely that more selective and potent NO modulators (NOS inhibitors and NO donors) given on a regular basis may have clinical benefit. Besides the determination of the exact role of different NOS enzymes and other sources of NO in airways, NO modulators will be needed to assess their potential clinical use.

Nitric Oxide and Reactive Nitrogen Species in Asthma and Chronic Obstructive Pulmonary Disease

Exhaled NO [1••,7] and iNOS mRNA in airway epithelial and inflammatory cells [2] are increased in asthma.

Exhaled NO in stable COPD [8] is lower than in either smoking or nonsmoking individuals with asthma, despite abundant iNOS and nitrotyrosine-positive sputum cells in COPD patients as compared with healthy smokers [9]. This may be due to the fact that tobacco smoking downregulates iNOS and decreases endothelial arginine content [10], in addition to the depletion of NO by formation of peroxynitrite, as the results of oxidative stress in COPD.

Severe airway inflammation, prevalence of neutrophilic inflammation, oxidant/antioxidant imbalance, and high iNOS presence in sputum cells [9], alveolar macrophages, alveolar walls, bronchial epithelium, and vascular smooth muscles of COPD patients [9,11] outweighs the effect of smoking on exhaled NO [12].

We have demonstrated that peripheral airways/alveolar region is the predominant source of elevated exhaled

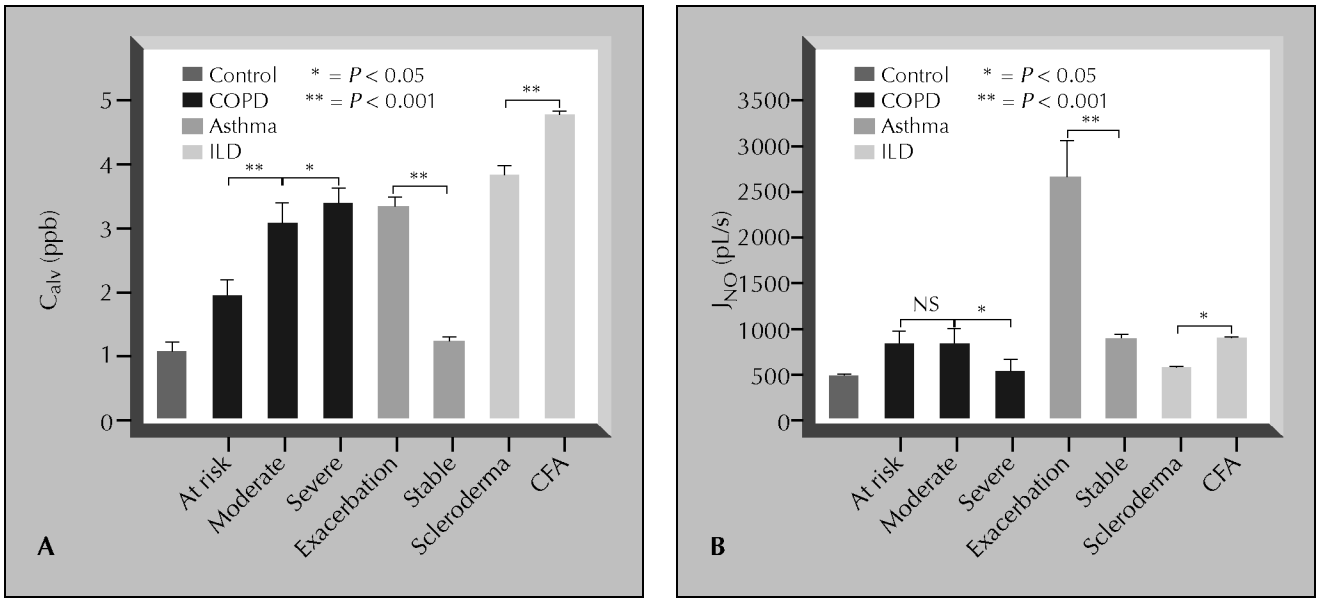

Figure 1. Small airway (**A**) versus bronchial (**B**) exhaled nitric oxide (NO) measurements in asthma, chronic obstructive pulmonary disease (COPD), and interstitial lung disease (ILD). CFA—crytogenic fibrosing alveolitis. (*Adapted from* Paska *et al.* [11].)

NO in COPD (Fig. 1*A*). In contrast, increased exhaled NO levels in asthma (Fig. 1*B*) are mainly of larger airways/bronchial origin [13]. Prevalence of alveolar-derived NO in COPD is possibly related to the iNOS in macrophages, alveolar walls, and bronchial epithelium of COPD patients [11].

Although the counts of nitrotyrosine-positive cells correlate with the severity of airway obstruction in COPD [9], it is still unclear whether nitrotyrosine is merely a biomarker of reactive nitrogen species (RNS), or whether it actively contributes to cellular dysfunction and development of the airway inflammatory processes in COPD.

In this respect, the recent discovery of apparent enzymatic "nitrotyrosine dinitrase" activity in rat spleen and lung homogenates [14] may further hint at the potential significance of nitration (and dinitration) as a signaling mechanism.

Diverse Effects of Nitric Oxide

The complexity of NO as a physiologic messenger and cytotoxic or cytoprotective effector molecule is based on its biosynthesis, which includes transcriptional, translational, and post-translational regulatory mechanisms; its concentration; its site of production; and its association with other molecules or proteins.

NO binds to several different proteins—for example, cytochrome P450, the primary O_2-reducing protein, and inhibits its activity without being reduced [15]. This process allows O_2 to be reduced exclusively in the presence of NO but independently of NO, explaining, perhaps, the existence of discrepancies between the levels of oxidative stress and exhaled NO in more severe asthma [16] and COPD [12].

Nitration of proteins

Reactive nitrogen species modify proteins in asthma and COPD, and the magnitude of this modification correlates with the degree of oxidative and nitrosative stresses. Protein nitration is unique among post-translational modifications in its dependency on reactivity of tyrosine residues in the protein target. It may be achieved by peroxynitrite (formed from the reaction between NO and O_2^-), through the reaction of NO with protein tyrosyl radicals, or by the reaction of nitrite with peroxidases [17].

Nitration of proteins by peroxynitrite is a dose-dependent process related to formation of two distinctive forms of nitrated proteins: stable 3-nitrotyrosine (nitration) and labile S-nitrosocysteine (S-nitrosation) [18]. Both of these nitrated proteins can be further enzymatically modified by glutathione S-transferase or glutathione peroxidase via (1) converting NO_2^- to NH_2^- in tyrosine residues; (2) denitrating NO_2^- directly/indirectly in tyrosine residues; (3) changing S-nitrosothiol (SNO) to SH^- in cysteine residues; (4) or denitrosation.

Nitration of mitochondrial proteins may change the activity of several enzymes involved in energy production (glutamate dehydrogenase), in the electron transport chain (cytochrome oxidase and adenosine triphosphatase), or in energy distribution (creatine kinase) [19••]. In fact, both energy production and apoptosis are affected by NO and related oxides [20] and are different in asthma and COPD. The susceptibility of asthmatic bronchial epithelium to oxidant-induced apoptosis is greater than normal [21], but alveolar macrophages and bronchial epithelial cells from smokers have reduced cell death [22]. The activity of enzymes in the central bronchial epithelium that protect cells

against oxidative damage [19••] is low, owing to nitration in patients with COPD [23].

Protein nitration may be beneficial. Therefore, it has been shown that surfactant protein A (SP-A), a product of tyrosine nitration of human pulmonary surfactant, downregulates T cell–dependent alveolar inflammation and protects against idiopathic pneumonia injury [24]. Tyrosine nitration in proteins is also sufficient to induce an accelerated degradation of the modified proteins by the proteasome, which may be critical for the removal of nitrated proteins in vivo [25••].

Tyrosine

Tyrosine nitration, a covalent post-translational and reversible [26] protein modification, occurs under basal or inflammatory diseases and changes protein function. It may occur through either peroxynitrite-dependent (interaction of peroxynitrite either with O_2^- or CO_2) or peroxynitrite-independent pathway. The latter involves tyrosine nitration by myeloperoxidase [27], eosinophil peroxidase, tyrosyl radicals, or by reaction of NO with a tyrosyl radical [17].

Nitration of tyrosine residues in tyrosine kinase substrates may prevent phosphorylation and, therefore, inhibit tyrosine kinase function in cellular signaling [26]. This may represent a novel mechanism of NO interaction with tyrosine kinase signaling, although the inhibition of tyrosine phosphorylation by tyrosine nitration remains highly speculative.

Nitrotyrosine

Nitrotyrosine can be formed when nitrite, or NO, is oxidized to nitrogen dioxide or peroxynitrite that couples with the tyrosyl radical to form a weak complex, which re-aromatizes to nitrotyrosine. A peroxynitrite-independent mechanism of nitrotyrosine formation is through the direct reaction of NO with a tyrosyl radical [17], when NO can form an unstable complex with the tyrosyl residue of prostaglandin H synthase-2; this complex can be oxidized to form a nitrotyrosine. Alternatively, free tyrosine can act as a co-substrate in myeloperoxidase-mediated tyrosine nitration.

Nonasthmatic lungs show little or no nitrotyrosine staining, whereas lungs of patients who die of status asthmaticus have a high presence of nitrotyrosine in both the airways and lung parenchyma. We have identified an increased bronchial iNOS activity and nitrotyrosine formation in mild asthma [2] and elevated levels of free nitrotyrosine in exhaled breath condensate (EBC) during asthma exacerbations [28], although nitrotyrosine formation in airway epithelial and inflammatory cells is significantly higher in COPD than in asthma [9].

Despite the recent discovery of enzymatic "nitrotyrosine dinitrase" activity in lung homogenates [14], suggesting the potential role of nitration (and dinitration) as a signaling mechanism, it remains to be established whether nitrotyrosine is merely a biomarker of increased nitrosative stress or whether it actively contributes to cellular dysfunction and development of the airway inflammatory processes in asthma or COPD.

Prostacyclin synthase

Both NOS and prostacyclin synthase maintain a balance of vasodilators and vasoconstrictors. This balance may be disrupted by oxidative stress or by prostaglandin synthase nitration by peroxynitrite, resulting in reduced prostacyclin-mediated vasodilation [29] and elevated levels of proinflammatory prostanoids, such as PGE_2 and $PGF_{2\alpha}$, in EBC in COPD [1••], but not in asthma.

Pulmonary hypertension is a complication of severe COPD that is associated mostly with remodeling of the pulmonary arterial walls. Currently, prostacyclin derivatives, endothelin antagonists, and NO donors [30] have been effectively used in patients with primary pulmonary hypertension. We speculate that iNOS inhibitors may also be effective in the treatment of secondary pulmonary hypertension by reducing or reversing arterial wall and airway remodeling in COPD.

Surfactant

Nitration of pulmonary SP-A diminishes its protective role against the collapse of small airways. Therefore, it can be speculated that SP-A may have impaired function in chronic smokers and patients with COPD as a result of peroxynitrite formation.

It has been shown that men with alleles of loci flanking SP-B have more severe COPD (forced expiratory volume in 1 second/forced vital capacity ratio ≤40%) [31], indicating that the surfactant protein alleles may be useful in COPD by either predicting the disease in a subgroup or by identifying disease subgroups that may be used for therapeutic intervention.

Superoxide dismutase

Superoxide dismutase (SOD) activity is reduced in asthma and COPD. Reactive nitrogen species impair the crucial antioxidant enzymes, such as SOD, catalase (CAT), and glutathione peroxidase (GPX) in a donor-specific and dose-dependent manner [32]. Peroxynitrite specifically nitrates only one tyrosine residue, Tyr34, located near the bound manganese of SOD that may cause mitochondrial dysfunction in asthma and COPD.

S-nitrosylation

Nitrosylation is a chemical and not an enzymatically catalyzed reaction that depends on the local concentration of NO and superoxide radicals and heme proteins. There is some specificity in nitrosylation; however, not every protein with available cysteine residues becomes nitrosylated [26]. SNO protein levels may be influenced by the activity of either iNOS or constitutive neuronal NOS [33]. This suggests that SNO protein modification

may serve as a major effector of NO-related bioactivity within human lung, both in NOS-containing cells and during NO-derived intercellular signalling.

Expression of some regulatory proteins, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin mRNA, is 10 times lower in both bronchoalveolar lavage fluid cells and biopsy tissue in steroid naïve individuals with asthma versus healthy individuals [34]. NO can reversibly nitrosylate cysteine residue in the active site of the caspase enzyme [26], reducing cell survival, but treatment with the caspase inhibitor prevents septal cell apoptosis and emphysema development [35].

The delayed apoptosis of eosinophil in the bronchial mucosa in asthma and macrophages in COPD [22] has been indicated as a novel mechanism by which these cells accumulate in the airways. This reduced airway macrophage apoptosis in COPD is linked to cytoplasmic expression of p21 proteins, and their nitrosylation by NO results in activation of activity instead of inhibition.

Therefore, treatment with NO donors may restore the lack of tracheal SNOs, an endogenous bronchodilator, in acute severe asthma [36], or may restore the delayed cell apoptosis in COPD.

Histones

Chemical nitration of histones has been previously demonstrated in vitro and, recently, in vivo [37]. In NO-exposed histones, cytoplasmic proteins were nitrated after only 1 day of exposure, although nitration of histones was not apparent until 3 days, and then it increased with time, reaching a maximum at about 6 days [37]. Therefore, the delay in histone nitration may be due to relative inaccessibility of nuclei to the nitrating RNS, suggesting that histones are appreciably more stable than the average cellular protein, and their slower turnover may permit them to accumulate 3-nitrotyrosine more than high turnover proteins. Hence, the presence of nitrated histones in tissues may reflect the long-term exposure to RNS [16].

Inducible NOS and other NF-κB–dependent genes involved in inflammation may be regulated by the specific recruitment of histone deacetylases (HDAC)-2 to NF-κB at target promoters and the consequent effects on their acetylation status [38], which may further enhance cytokine induction of both the iNOS and the NF-κB.

HDAC activity and expression is reduced in bronchial biopsy samples obtained from mild asthmatics [39••] compared with normal subjects, but may be restored by steroids that activate histone acetyltransferases [39••]. It has been shown that in subjects with severe asthma and COPD with reduced HDAC activity, the ability of inhaled steroids to control inflammation may be lost [39••].

Nitric oxide Modulators

The effect of NO may be beneficial or deleterious and both NOS inhibitors and substrates of NOS could have great therapeutic potential in such steroid-resistant pathologic conditions as severe asthma and COPD.

Treatment with NO modulators in different redox states may result in different effects. For example, 3-morpholinosydnonimine (SIN-1), which produces both NO and superoxide radicals, may cause neurotoxicity, but nitroglycerin, which produces NO, has neuroprotective effects. It is clear that when cellular conditions favor nitrosylation, NO has positive effects; however, if NO is produced under conditions in which there is also superoxide radicals production, NO can be toxic [26]. We have shown that further increase in NO in mild asthmatics after inhalation of L-arginine was not associated with any deterioration of their asthma symptoms and lung function [40].

Nitric oxide donors

There are several reasons for the considerable interest in the development of new compounds to act as NO donors for patients with lung diseases.

NO plays an important role in bactericidal activity in the lungs, ciliary beating, and mucociliary dysfunction [41]. Low levels of exhaled interferon-gamma (IFN-γ) [42] and suppression of endogenous NO production by corticosteroids in asthma [7], or by smoking in COPD [12], might contribute to the recurrent chest infections in these patients. Therefore, an anti-inflammatory treatment (for example, a combination of corticosteroids and NO donors) that preserves NO production may be optimal in asthma and COPD.

Rhinovirus infection, which primes the cytokine and histamine production from mast cells and basophils, as well as the defective type 1 immune response to rhinovirus in atopic asthmatic individuals, may be implicated in the pathogenesis of virus-induced exacerbations of asthma [43]. Elevated levels of exhaled NO in subjects with upper respiratory infection [44] may inactivate a protease enzyme reaction in the human rhinovirus, possibly via transnitrosation, affecting both bacteria and viruses [45].

Therefore, we speculate that NO donors may also have a potential role in the treatment of the common cold by restoring the antiviral effect of NO, especially in atopic asthmatics, who suffer from frequent lower-respiratory-tract (LRT) infections and have more severe and longer-lasting LRT symptoms than normal subject [43]. Perhaps these patients will benefit from a course of oral NO donors at the time of potential or current viral infection.

The fact that inflammatory response in COPD, or in severe steroid-resistant asthma, is essentially steroid-resistant triggered the development of more effective antioxidants, NO donors, and NOS inhibitors. Oxidative stress occurring in COPD and asthma can be reduced by antioxidants, including NO per se, by virtue of the scavenging action of its hydroxyl radicals (OH), following Fenton chemistry. It can also be speculated that oxidative stress, which promotes NO inactivation by ROS leading to functional NO deficiency and wide-

spread accumulation of protein nitration products, can be ameliorated with anti-oxidants or NO modulators, which will enhance NO availability.

There is an advantage of NO donors over antioxidants, especially in COPD, as the latter may be able to reduce an oxidative stress in these patients, but will not be able to restore their NO deficiency owing to the effect of smoking. Therefore, markedly impaired endothelial and host defense function may persist with antioxidant treatment alone.

Resveratrol, a naturally occurring polyphenol that possesses some antioxidant and cardioprotective properties linked to the induction of the iNOS expression [46], may be used to normalize the reduced apoptosis of bronchial epithelial cells from smokers and COPD patients in an NO-dependent manner.

Inhaled nitric oxide donors

Pulmonary hypertension is a common complication of COPD and is linked to low levels of exhaled NO and to extensive remodeling of the pulmonary arterial walls. Inhaled NO, as a powerful vasodilator, has positive effect in patients with pulmonary hypertension and may be useful in severe COPD patients with cor pulmonale. Methahemoglobinaemia, however, and the build-up of toxic levels of nitrite (NO_2^-) limits its use in clinical practice. Sildenafil, an effective and selective pulmonary vasodilator, may be superior to inhaled NO because it increases cardiac output and does not increase wedge pressure [47].

Inhaled O-nitrosoethanol gas, as a novel alternative means of providing NO bioactivity, has been successfully used in the treatment of persistent pulmonary hypertension of newborns [48].

S-nitrosothiols and other nitric oxide–generating compounds

S-nitrosothiols (RSNOs), which occur endogenously and are reduced in acute asthma [36], release NO both enzymatically and nonenzymatically, and have been administered to humans in small clinical trials. One of the main problems with the use of RSNOs is their unpredictable rate of decomposition by enzymatic degradation and as a result of transnitrosation [49]. Commercially available RSNOs, S-nitroso-*N*-acetylpenicillamine (SNAP), *N*-acetyl-S-nitroso-penicillaminyl-S-nitrosopenicillamine and S-nitrosoglutathione (GSNO), or diazeniumdiolates compounds, which generate NO spontaneously [49], have not yet been used therapeutically in humans.

Albumin is an abundant circulating protein that, in its nitrosated form, effectively acts as a reservoir of NO. Different forms of poly-SNO-albumin have been prepared by covalent modification of albumin prior to nitrosylation [50], but many aspects of their biochemistry that need to be addressed before their therapeutic potential can be fully realized.

Nitric oxide synthase substrates

Most substrates of NOS are arginine or NG-hydroxy-L-arginine derivatives (NOHA). This suggests that the α-amino acid portions of those substrates play an important role in the catalysis. Because some simple guanidines, such as aminoguanidine, are strong NOS inhibitors through binding at the active site, it appears that the α-amino acid moiety of arginine can be removed without detrimental consequences, whereas the integrity of the guanidine function must be partially retained [51••].

This assumption that simple exogenous compounds bearing guanidine or hydroxyguanidine functions, but without an amino acid function, could be oxidized by NOS in a manner similar to NOHA, with a significant rate of NO formation, has recently lead to the discovery of novel substrates for NOS. Some of these novel simple *N*-aryl-*N*'-hydroxyguanidines might be used as selective NO donor supplementation for iNOS [51••]. This finding is significant not only for understanding the NOS mechanism, but also in using such compounds as isoform-specific probes in biomedical experiments.

L-arginine supplementation has been studied in a variety of clinical situations in which the increase of NO production is desired. For example, orally administered [52] and inhaled L-arginine [41] has been used in normal subjects and patients with primary ciliary dyskinesia to improve the bactericidal activity of the lungs, ciliary beating, and mucociliary dysfunction. L-arginine coated endovascular stents have been tested in controlling restoration of blood flow after balloon angioplasty to control restenosis, either alone or as a pharmaceutical adjunct to a vascular device [53].

There are several reasons to consider the use of L-arginine as a treatment in COPD. Cigarette smoke reduces constitutive pulmonary artery eNOS activity and possibly decreases endothelial arginine content, as L-arginine supplementation increases serum and endothelial L-arginine stores and prevents smoke-induced endothelial dysfunction [10]. Besides increasing the formation of NO, L-arginine has substantial concentration-dependent antioxidant properties [54], which are based on the guanidinium group rather than on the α-amino carboxylic acid structure. Therefore, the mechanism by which L-arginine protects against oxygen radical-induced airway injury in asthma and COPD may be its antioxidant properties, owing to a chemical moiety different from that necessary for NO biosynthesis. The antioxidant properties of L-arginine might result not only in increased formation of NO but also in a higher bioavailability of NO, because L-arginine stabilizes the NOS homodimer needed for the formation of NO and delays the cell-mediated breakdown of NO [55].

Nitric oxide synthase inhibitors

Arginine competitors seem to be promising targets. We have used some of the early relatively nonselective inhibitors of

arginine analogues, such as structural analogues of L-arginine, for example N^G-methyl-L -arginine (L-NMMA) and N^G-monomethyl arginine methyl ester (L-NAME) [56]. Nebulized L-NMMA and L-NAME both reduce exhaled NO [56] in asthmatic patients, although this is not accompanied by any changes in lung function, including during the early and late asthmatic responses alter allergen-induced bronchoconstriction in either single or dual responders [57].

Aminoguanidine, a more selective inhibitor of iNOS, reduces exhaled NO in asthmatic patients, but has little effect in normal subjects [58], indicating that iNOS is an important source of the increased exhaled NO in asthma. Recently, a series of 5,6-dihydropyridin-2-imines, new selective inhibitors of human iNOS (hiNOS), have been synthesized and biologically evaluated [59]. We have reported a profound (>90% reduction in asthma), rapid (within 15 minutes), and sustained (longer than 72 hours) reduction in exhaled NO down to, presumably constitutive NO levels, fewer than 2 ppb in both normal and asthmatic subjects after a single oral dose of a selective iNOS inhibitor L-NIL [60].

Under conditions of inflammation and oxidative stress, superoxide anion will preferentially react with available NO rather than its endogenous neutralizer superoxide dismutase, thus increasing the formation of peroxynitrite in tissues [29]. Therefore, the use of iNOS inhibitors by reducing "available" NO may restore the preferred pathway of superoxide radicals detoxification, via superoxide dismutase.

We suggest that endogenously produced "low levels of NO" might have a protective role in maintenance of structural integrity of pulmonary tissue after smoke-induced damage. The expression of eNOS in alveolar macrophages, endothelial, or bronchiolar epithelial cells suggests that eNOS plays an additional role, besides iNOS, in the NO housekeeping in inflammatory processes in pulmonary tissue in patients with emphysema. Therefore, NO donors may be beneficial in patients with COPD to restore eNOS-derived NO, as well as the specific iNOS inhibitors, which will reduce the iNOS expression in COPD, and may also protect and scavenge electrolysis-generated superoxide radicals.

This may provide the rationale for simultaneous treatment of COPD and severe asthmatics with NO donors and iNOS-specific inhibitors.

Modification of tyrosine nitration

Several drugs can modify or restore nitrated proteins. For example, antioxidants t-butylhydroxytoluene reverse impaired smooth muscle sarcoplasmic and endoplasmic reticulum Ca^{2+}-adenosine triphosphatase (SERCA) by reducing tyrosine-nitrated SERCA protein [61].

Methods to Sample and to Measure NO, RNS, and Protein Nitration In Vivo

Exhaled breath analysis

The need to monitor inflammation in the lungs has led to the exploration of exhaled gases and condensates, which may assist in differential diagnosis of pulmonary diseases, assessment of disease severity, and response to treatment [1••].

Exhaled nitric oxide

Nitric oxide is the most extensively studied exhaled marker, and abnormalities in exhaled and nasal NO have been documented in several lung diseases, particularly asthma [62]. Exhaled NO and nitrite/nitrate levels in breath condensate can be used to monitor dose-dependent onset and duration of action of corticosteroids [3], and they are valuable parameters to monitor complex NO biochemistry in the clinic.

An interesting method of measuring exhaled NO at several exhalation flow rates has recently been described [1••]. Peripheral airways/alveolar region may be the predominant source of elevated exhaled NO in COPD, whereas increased exhaled NO levels in asthma are mainly derived from the larger airways [13]. This novel technique can be used to monitor not only the disease but the effect of treatment with NO modulators, which may have different mechanisms and sites of their actions.

Exhaled breath condensate

Exhaled breath condensate is collected by cooling or freezing exhaled air and is totally noninvasive. Although the collection procedure has not been standardized, there is strong evidence that abnormalities in condensate composition may reflect biochemical changes of airway-lining fluid [62]. Potentially, EBC can be used to measure the targets of modern therapy in clinical trials and monitor asthma and COPD in clinic.

A significant proportion of NO is consumed by chemical reactions in the lung leading to formation of nitrite, nitrate, and S-nitrosothiol in the lung epithelial lining fluid. In contrast to decreased S-nitrosothiols in tracheal aspirates from children with asthmatic respiratory failure [36], increased nitrotyrosine in asthmatic airway epithelium has been inferred from immunostaining of lung biopsies [2], and elevated levels of free nitrotyrosine have been observed in EBC in asthma [16].

Measurement and identification of proteins in exhaled condensate is controversial [62], although higher concentrations of total protein in exhaled condensate have been found in young smokers compared with nonsmokers [63]. Various proteins derived from airways and unlikely to be contaminated with saliva have been detected in EBC by two-dimensional electrophoresis [64]. Although their range and source are still unclear, the proteins recovered in EBC might be used to noninvasively monitor respiratory diseases in the future.

Exhaled temperature

Asthma is characterized by vascular hyperperfusion, which is reflected by elevated exhaled temperature, which may be an index of airway inflammation [1••]. In contrast, low

exhaled temperature in COPD may reflect reduced endothelial NO release and impaired pulmonary circulation. Exhaled temperature, therefore, may be used for monitoring pulmonary circulation, as well as the effect of NO modulators in asthma and COPD.

Gas chromatography/mass spectrometry and enzyme immunoassays techniques

Nitrotyrosine and tyrosine can be simultaneously detected by HPLC, which measures either the specific UV absorbance of amino acids, the specific fluorescence of nitrotyrosine-fluorescence derivates, or the electrochemical properties of nitrotyrosine. None of these approaches, however, are capable of measuring nitrotyrosine in proteins or peptides.

Although an enzyme immunoassay (EIA) method combines sensitivity and specificity with the ability to process several specimens to quantify nitrotyrosine, the proteomics approach may be preferential to enrich the samples, because nitrotyrosine and protein content is very low in EBC [28,65].

Specific anti-nitrotyrosine antibodies (immunoprecipitation) can be used to detect nitrotyrosine by immunohistochemistry—for example, in nasal mucosa of patients with allergic rhinits [66]—although Western blotting on a two-dimensional gel, followed by mass spectrometry [19••] may offer higher sensitivity. Immunoprecipitation has certain limitations, because it is limited to proteins for which specific antibodies are available, and it cannot identify which specific tyrosine residues have been nitrated.

A combination of mass spectrometric techniques allows the identification of nitrated proteins in complex protein mixtures from tissue samples [37], and an electron capture-negative chemical ionization–gas chromatography/mass spectrometry (EC-NCI GC/MS) is 100-fold more sensitive than liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-MS/MS) for analyzing 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in vivo [67].

Proteomic approach

Many properties of proteins (*eg*, interactions, post-translational modifications) cannot be predicted from DNA sequence (genomics). Although mass spectrometry allows the identification of proteins from crude cell extracts after two-dimensional electrophoretic separation, it is difficult to measure low-abundance proteins of interest in the presence of a large excess of relatively abundant proteins. Therefore, for effective proteome analysis, it is critical to enrich the sample to be analyzed in subfractions of interest (proteomics). The precision of proteomics for identifying nitrated proteins is based on two factors: the use of two-dimensional gels to separate cellular macromolecules and the ability to obtain highly accurate peptide molecular mass values to identify immunoreactive proteins.

Proteomics has the potential for identifying novel targets of tyrosine nitration in cells and tissues to obtain a view of the nitro- [19••] and phosphoproteome, or to identify proteins undergoing *S*-nitrosylation in vivo. Recently, a proteomic approach identified more than 40 nitrotyrosine-immunopositive proteins, including 30 not previously identified, which became modified as a consequence of the inflammatory response [19••]. These targets include proteins involved in oxidative stress, apoptosis, adenosine triphosphate (ATP) production, and other metabolic functions.

We have identified low levels of proteins in EBC, which are distinctive from saliva proteins (unpublished data). Proteomics allowed it to detect proteins that undergo endogenous nitration and, therefore, to eliminate artifacts that may arise when exposing breath condensate to exogenous NO or nitrating agents. Therefore, proteomics to detect exhaled breath proteins promises to be a sensitive means to initiate clinical studies in asthma and COPD on mechanisms, selectivity, and consequences of biologic tyrosine nitration.

Conclusions

Selective and more potent NOS inhibitors and NO donors, as well as noninvasive clinical methods to assess NO biochemistry, will lead to a better understanding of its deleterious and beneficial effects and novel treatments in COPD and asthma patients.

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