

Reactive nitrogen species in refractory asthma: Markers or players?

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Refractory asthma is any form of severe asthma that is poorly controlled despite the use of high doses of medications, especially inhaled corticosteroids.¹ It is thought to affect a minority of all asthmatic patients and yet accounts for an excessive share of the morbidity and socioeconomic costs of the disease.¹ Improving our understanding of the mechanisms involved in refractory asthma and thereby developing new treatment strategies is a high priority. Currently, the pathologic abnormalities of refractory asthma are not well defined but generally involve the following features: increased eosinophils, lymphocytes, and helper T_H2 cytokines, which are resistant to corticosteroid therapy; increased infiltration of neutrophils; and evidence of significant airway remodeling.¹ Whether these features simply represent the severe end of the pathologic spectrum of asthma or are unique to refractory asthma *per se* is not known.

One marker of airway inflammation that is increased in patients with asthma is exhaled nitric oxide (NO). Exhaled NO levels correlate well with eosinophilic inflammation in sputum, increase during asthma exacerbations, and decrease with treatment with corticosteroids.² This exhaled NO is thought to originate mainly from inducible NO synthase (iNOS), the expression of which is also suppressed by corticosteroid therapy.³ Persistently increased exhaled NO levels have been documented in patients with severe asthma, despite treatment with oral corticosteroids.⁴ This led Sugiura et al,⁵ in their article in this issue of the Journal, to speculate that NO or other reactive nitrogen species (RNS) might contribute to the pathogenesis of refractory asthma.⁵

When NO is formed in the airways, it can undergo a series of conversions to produce the end products nitrite and nitrate, as well as S-nitrosothiols, a storage form of functionally active NO. During these conversions, highly reactive RNS can be produced that can react with amino acids, lipids, and DNA, reflecting a milieu that has become known as *nitritative stress*.² One marker of nitritative stress is 3-nitrotyrosine (3NT), which occurs after attack of protein tyrosine residues by nitrogen dioxide or molecules with similar reactivities. Nitritative stress is thought to contribute to the pathophysiology of various disease states, including asthma. In particular, RNS have been demonstrated in the airways

of asthmatic subjects.^{3,6-9} In addition, experimental models of airway inflammation have suggested that RNS might contribute to many of the manifestations of asthma, such as airways hyperresponsiveness,¹⁰ microvascular permeability,¹¹ and airway remodeling.¹²

To determine whether excessive nitritative stress is involved in severe refractory asthma, Sugiura et al⁵ examined the exhaled air and sputum of patients with refractory asthma for markers of nitritative stress, including exhaled NO, iNOS expression, and 3NT formation in sputum leukocytes. They also measured xanthine oxidase (XO) activity, an enzyme known to contribute to oxidative stress, in sputum supernatants. The levels of markers were compared with those from healthy control subjects and subjects with well-controlled asthma. The researchers found significantly increased levels of exhaled NO and iNOS and 3NT-positive cells and XO activity in the samples from the patients with refractory asthma compared with those from the other 2 groups. There were significant correlations between 3NT-positive cells and XO activity and iNOS expression. Moreover, there were significant correlations among all 4 markers and lung function, as measured by using FEV₁. As in other studies of severe asthma, there were increased numbers of neutrophils in the sputum from patients with refractory asthma compared with that from the well-controlled and healthy groups. Patients with both well-controlled and refractory asthma had increased eosinophils in their sputum compared with the healthy control subjects. The authors concluded that RNS might be involved in the pathogenesis of refractory asthma.

The results of this study provide strong circumstantial evidence for nitritative stress in human subjects with refractory asthma. The association of 3NT with sputum leukocytes suggests localized oxidative events drive RNS formation. These events most likely involve reduced nicotinamide adenine dinucleotide phosphate oxidase and neutrophil peroxidases, eosinophil peroxidases, or both (ie, myeloperoxidase and eosinophil peroxidase, respectively), which are known to be capable of catalyzing oxidation and nitration of various biologic targets.¹³ Interestingly, Sugiura et al⁵ propose an alternative mechanism of RNS formation, demonstrating that patients with refractory asthma had increased oxidative stress, as indicated by increased sputum XO activity, which might provide a local source of superoxide anion and other reactive oxygen species. However, it is not clear how extracellular XO would be responsible for the formation of 3NT, which is primarily associated with leukocytes. Unfortunately, the authors did not address the cellular source of XO, which might originate from leukocytes but also from the airway epithelium. Based on seminal studies by Phan et al¹⁴ indicating that activated neutrophils can cause the activation of XO within endothelial cells through oxidative or proteolytic mechanisms, it could be argued that the increased activation of neutrophils or eosinophils in patients with refractory asthma might similarly be responsible for increased sputum XO activity. This is especially intriguing given the consistent finding of increased neutrophilia in refractory

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asthma and suggests a unique pathophysiologic role for the neutrophil and XO in this form of the disease.

It is intriguing that the markers of nitrative stress in the study by Sugiura et al⁵ correlate closely with lung function, suggesting a potential mechanistic link to the asthma phenotype. This is especially noteworthy because lung function and inflammation are often poorly correlated in asthma. However, a causal role of RNS in the pathophysiology of refractory asthma cannot be determined from this study. In particular, it is not possible to know whether RNS contribute to the mechanisms involved in refractory asthma or whether they are simply formed in excess because of the underlying inflammatory process. In fact, because activated leukocytes might be responsible for oxidative chemistry that promotes nitration, as well as the activation of XO,¹⁴ observations of significant increases of neutrophils within sputum from patients with refractory asthma might simply suggest that the increased levels of 3NT or XO activity reflect the presence of activated leukocytes. Although the authors did not attempt to measure these, it is likely that other oxidative or inflammatory markers might similarly be increased in association with the presence of activated sputum leukocytes. Therefore in spite of the intriguing correlations between markers of nitrative stress and lung function, it would be premature to directly implicate RNS in disease pathology based on the current findings.

Despite extensive investigation, the specific contribution of NO, RNS, or both to the pathophysiology of asthma is unclear because the biochemical pathways for metabolism of NO within the lung are complex and not fully understood.^{13,15} If NO were playing an important role in the pathophysiology of asthma, then reducing NO production through inhibition of iNOS would be expected to result in decreased airway inflammation or airways hyperresponsiveness. However, a recent study in human subjects demonstrated that inhibition of iNOS in subjects with asthma had no such effects.¹⁶

One aspect of NO biochemistry that appears to be markedly altered during asthma is the presence of S-nitrosothiols, which might represent a storage form of NO but might also regulate lung function and inflammation through mechanisms independent of NO itself. S-nitrosothiol levels have been shown to be reduced in patients with asthma,^{8,17} but other investigators have reported increased levels.¹⁸ Unfortunately, Sugiura et al⁵ did not address potential alterations of S-nitrosothiol levels in refractory asthma. Because S-nitrosothiols might provide protective functions in asthma, including bronchodilation, downregulation of inflammation by inhibition of nuclear factor κ B activity,¹⁹ and modulation of airway remodeling through inhibition of matrix metalloproteinase 9,²⁰ reduction of S-nitrosothiol levels might contribute to asthma pathogenesis. Recently, data from Que et al²¹ have suggested that the loss of S-nitrosothiols in the airways of asthmatic patients might be due to an increase in the activity of S-nitrosoglutarathione (GSNO) reductase, which metabolizes GSNO to form ammonia and oxidized glutathione. GSNO reductase appears to be an important regulator of bronchial tone based on observations that mice lacking GSNO reductase are protected against airways hyperresponsiveness associated with allergic inflammation.²¹ Interestingly, polymorphisms of the gene encoding GSNO reductase have been linked to human asthma.²² It will be important to unravel to what extent GSNO reductase is differentially expressed between patients with mild and those with refractory asthma in addition to the frequency of polymorphic variants of GSNO reductase in these different groups.

Despite the lack of specificity of the findings of Sugiura et al,⁵ the results are compelling because many of the features that define refractory asthma might be linked to changes in redox homeostasis, as reflected by nitrative stress. As pointed out by the authors, these features include steroid resistance, persistent eosinophilic inflammation, increased neutrophilia, and airway remodeling. The picture is emerging that asthma might involve a loss of beneficial NO (in the form of S-nitrosothiol) in association with oxidation of NO to highly reactive nitrogen species that reflect nitrative stress. The challenge for investigators will be to understand the chemical environment and the players that create this environment. XO measured by Sugiura et al⁵ might be one of these players, but other factors, such as airway pH, loss of antioxidant defenses, and redox state of other compounds, such as thioredoxin, also warrant additional consideration. Further investigations to determine cellular sources and molecular targets of RNS and interventional studies to specifically block the action of RNS are clearly needed to unravel the intricacies of NO and its derivatives in normal airway biology and pathophysiology.

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