Exhaled Nitric Oxide Is Elevated in Patients With Progressive Systemic Sclerosis Without Interstitial Lung Disease*

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**Background:** Progressive systemic sclerosis (PSS) is a multisystem disorder of unknown etiology. Interstitial lung disease (ILD) is a major cause of mortality in this condition, and a major challenge in this regard is to identify parameters that would predict the onset or progression of ILD in patients with PSS. Abnormal cellularity of BAL fluid (BALF) has been demonstrated in patients with PSS without ILD.

**Study objectives:** We investigated exhaled nitric oxide (NO) as a noninvasive marker of pulmonary inflammation in patients with PSS with and without clinical and radiologic evidence of ILD. This was compared with the cellularity of BALF. Our hypothesis was that exhaled NO was elevated in patients with PSS without ILD who had abnormal BALF cellularity.

**Setting:** Pulmonology and rheumatology units of a university-based, tertiary referral hospital in Durban, South Africa.

**Study methods:** Exhaled NO was measured using a chemiluminescence analyzer in 12 patients with PSS and ILD and in 12 patients without clinical or radiologic evidence of ILD and in 30 healthy control subjects. BAL was performed in patients with PSS with and without the presence of ILD and in six healthy control subjects.

**Results:** Subclinical inflammation was confirmed by increased inflammatory cell counts in BALF from patients with PSS without ILD. Exhaled NO (mean [SEM]) was elevated in patients with PSS without ILD at 9.6 (0.7) parts per billion (ppb) compared to patients with PSS and ILD at 6.2 (0.6) ppb (p < 0.001) and healthy control subjects at 6.3 (0.2) ppb (p < 0.001).

**Conclusion:** Exhaled NO may therefore be an important noninvasive surrogate marker of inflammation in patients with PSS without ILD.

**Key words:** BAL; interstitial lung disease; nitric oxide; systemic sclerosis

**Abbreviations:** BALF = BAL fluid; DLCO = diffusion capacity of the lung for carbon monoxide; ILD = interstitial lung disease; iNOS = inducible nitric oxide synthase; NO = nitric oxide; NOS = nitric oxide synthase; ppb = parts per billion; PSS = progressive systemic sclerosis

Progressive systemic sclerosis (PSS) is a multisystem disorder of unknown etiology. It is characterized by fibrosis of the skin, blood vessels, and organs like the GI tract, lung, heart, and kidneys.1,2 Interstitial lung disease (ILD) occurs in 35 to 40% of patients with PSS and is the most significant cause of mortality in patients with PSS.3 There is ongoing pulmonary inflammation in patients with PSS without clinical or radiologic evidence of ILD as demonstrated by an abnormal BAL fluid (BALF) finding.3 This has led to an ongoing debate as to whether the inflammatory infiltrate precedes, occurs concurrently with, or follows the fibrotic process of ILD in patients with PSS. A major challenge in this regard is to identify parameters that would predict the onset or progression of ILD in patients with PSS.

Nitric oxide synthase (NOS), the enzyme responsible for the generation of nitric oxide (NO), exists in three major isoforms.4 Neuronal NOS and endothelial NOS are expressed constitutively and are responsible for NO production in the basal physiologic state. Inducible NOS (iNOS) is characteristically regulated by cytokines that increase NO concentrations in the inflammatory response.4 The formation...
of NO from the oxidation of L-arginine to citrulline occurs in many sites in the lung where it has different functions, including vascular tone regulation, proinflammatory and anti-inflammatory effects, neurotransmission, and tumor cell lysis.\textsuperscript{5} Studies have shown that exhaled NO is derived from both the upper and lower respiratory tract and may be a noninvasive surrogate marker of airway inflammation, particularly in asthma.\textsuperscript{6} Exhaled NO was measured in patients with PSS and ILD. It was elevated in patients with PSS and ILD,\textsuperscript{7} suggesting that infiltrating inflammatory cells at the sites of lung injury upregulated NO, thus increasing exhaled NO. Kharitonov et al\textsuperscript{8} found that exhaled NO was raised in patients with PSS and ILD but was decreased in patients with PSS and pulmonary hypertension when compared to healthy control subjects. This suggested that endothelial dysfunction inhibited the formation of NO in this cohort of patients.

To our knowledge, there are no studies evaluating exhaled NO in patients with PSS without ILD. As a result of the subclinical inflammation in patients with PSS without ILD, we postulated that exhaled NO would be elevated in these patients and may be an important noninvasive marker for the onset and progression of ILD in patients with PSS.

The aim of our study was to measure exhaled NO in patients with PSS with and without ILD and to correlate these levels with conventional parameters of disease activity. We also examined the effect of therapy on exhaled NO in this condition. Pulmonary inflammation in patients with PSS without ILD was confirmed by investigating the cellularity of BALF in these patients.

**Materials and Methods**

**Subjects**

Patients with PSS with and without clinically and radiologically evident ILD were studied.

**PSS in Patients With ILD**

The study comprised 12 patients (10 female patients); mean (SEM) age was 43 (12) years. Nine patients were Indian Asian, and three were African. All patients fulfilled the preliminary criteria of the American Rheumatism Association criteria for the diagnosis of PSS.\textsuperscript{1} None of the patients were smokers, and three were ex-smokers (stopped smoking 2 years ago). Patients with ILD had bilateral, fine, mid-to-late inspiratory crackles, and evidence of pulmonary hypertension on clinical examination. Evidence for pulmonary hypertension included a loud pulmonary component of the second heart sound, a left parasternal heave, and tricuspid regurgitation. This was confirmed by ECG evidence of right ventricular strain or hypertrophy and a mean pulmonary artery pressure of >30 mm Hg with Doppler echocardiography. Raynaud’s phenomenon and skin and joint involve-

ment were present in all patients. Six patients had esophageal dysmotility determined by barium swallow studies. The urea and electrolyte levels were normal. Chest radiographs in all patients showed a bilateral reticular nodular shadowing involving predominantly the lower lobes. High-resolution CT findings were typical of ILD associated with PSS and revealed heterogeneous areas of both alveolitis and honeycombing predominantly in the lower lobes. Spirometric lung function tests demonstrated a restrictive defect in all patients. The FEV\textsubscript{1} was 60 (17)% predicted; FVC, 63 (12)% predicted; and FEV\textsubscript{1}/FVC, >100% (Tiiffeneau index). The diffusion capacity of the lung for carbon monoxide (DL\textsubscript{CO}) was 60 (11) % predicted. The DL\textsubscript{CO} was measured using the single-breath method, and the values were corrected for measured hemoglobin and expressed as percentage of predicted values. ECG and echocardiography revealed no evidence of pulmonary hypertension. Transbronchial biopsy was performed to exclude other abnormalities and demonstrated interstitial inflammation in all patients. Open-lung biopsy was not performed on any patient since the clinical, radiologic, and transbronchial biopsy studies strongly suggested ILD associated with PSS, obviating the need for an open-lung biopsy in these patients.

All patients were commenced on therapy since they had disabling respiratory symptoms and impaired lung function. The patients were commenced on a regimen of azathioprine, 2 mg/kg/d, and low-dose prednisone, 20 mg/d tapered to 10 mg/d over 6 weeks. Monitoring of patients was done monthly using their symptoms and lung function test results to measure the response to therapy.

**Patients With PSS Without ILD**

Twelve patients (10 female patients; mean age, 49 [18] years) with PSS without evidence of ILD were recruited. Ten patients were Indian Asian, and 2 patients were African. None of the patients were smokers, but two were ex-smokers (stopped smoking 18 months ago). All patients had Raynaud’s phenomenon and skin and joint involvement. There was no clinical evidence of pulmonary hypertension. Eight patients had esophageal dysmotility determined by barium swallow studies. ECG and echocardiography detected no evidence of pulmonary hypertension. Their chest radiograph and high-resolution CT findings were normal. Spirometric lung function testing showed a FEV\textsubscript{1} of 94 (17)% predicted; FVC, 98 (10)% predicted; and DL\textsubscript{CO}, 70 (11)% predicted. Proton pump inhibitors and prokinetic agents were used to treat reflux esophagitis.

Healthy control subjects comprised 30 volunteers (16 male subjects) for exhaled NO and 6 volunteers (3 male subjects) for BAL. The subjects were matched for age, gender, and race. They were nonatopic as determined by history and nonresponsiveness to skin prick testing using common allergens (house dust mite, grass pollen, cat dander, and Aspergillus fumigatus); a positive finding was a wheal >3 mm. All were nonsmoking subjects aged 42 (3.8) years, with no history of lung disease. None had respiratory tract illnesses in the 6 weeks preceding the study. Healthy control subjects had normal spirometric lung function values: FEV\textsubscript{1}, 98 (8)% predicted; FVC, 99 (8)% predicted; and DL\textsubscript{CO}, 97(10)% predicted.

**Study Protocol**

At entry, lung function and serologic tests were performed and exhaled NO was measured. Four days after visit 1, exhaled NO was measured to evaluate the reproducibility of exhaled NO in patients with PSS. BAL was also performed at this visit. Lung function tests including histamine provocation testing, serologic tests, exhaled NO, and BAL were repeated following 6 months of therapy with oral prednisone and azathioprine in patients requiring therapy.
Exhaled NO Measurements

A Logan R2000 NO analyzer was used (Logan Research; Rochester, UK). This is a chemiluminescence analyzer that is designed for on-line recording of exhaled NO from 1 to 5,000 parts per billion (ppb) and a resolution of 0.3 ppb. It has a response time of < 0.55 s with high reproducibility. The analyzer has a feedback control unit that maintains pressure at 3 ± 0.4 mm Hg and flow at approximately 10 to 15 L/min (obstructed) and 5 L/min (unobstructed). These parameters do not affect the NO recording. The pressure changes in the mouthpiece and reaction chamber vary insignificantly and cause insignificant changes in NO reading (< 0.1 ppb). The sampling rate of the analyzer was kept constant at 220 mL/min for all measurements. The analyzer also measures CO2 (response time of 200 ms and resolution of 1% CO2).

Measurements were made with the subject sitting, at least 5 min before testing. The maneuver was started with exhalation to residual volume followed by rapid inhalation to total lung capacity. Thereafter, the patient exhaled slowly and steadily from total lung capacity over a 30-s period with an exhalation flow rate of 10 to 15 L/min into a wide-bore Teflon tube connected to the side-arm sampler of the analyzer. All subjects were allowed several dummy practice maneuvers. NO measurements were taken at maximum levels, mean levels, and at the level when the CO2 plateau was reached. The latter level represented the alveolar sample of NO as verified by studies in this area and is the value reported in the present study. Corrections for ambient NO levels were made with each measurement. The measurements of exhaled NO and the analyzer used were in keeping with the criteria set out by the European Task Force on the measurement of exhaled NO.*

Bronchoscopy and BAL

A bronchoscope (model F IT20D; Olympus; Tokyo, Japan) was inserted and wedged under adequate local anesthesia and sedation with midazolam in the right middle lobe. A segment was lavaged using three 60-mL aliquots of saline solution. The BAL samples were subjected to total cell counts and differential cell counts. A 1.8-mm size cup biopsy forceps was used to obtain transbronchial biopsy specimens from the right middle lobe segments.

Processing of BALF

The processing of BALF is described elsewhere.10 Briefly, the pooled BALF specimen was passed through sterile nylon gauze (Sigma Diagnostic; Poole, UK). Aliquots of 2 mL of lavage fluid were cytocentrifuged at 1,000 revolutions/min for 10 min. The cell pellet was resuspended in Hanks’ balanced salt solution. A 1.8-mm size cup biopsy forceps was used to obtain transbronchial biopsy specimens from the right middle lobe segments.

Statistical Analysis

Results are expressed as mean (SEM). The repeatability of measurements was examined by the intraclass correlation coefficient (R), as the proportion in the variance in the measurements to the true variance between subjects.11

Statistical analysis of comparisons between the study groups and healthy control subjects was performed using the Student’s t test for parametric data. A value of p < 0.05 was regarded as significant. Statistical analysis was done using the Statistical Analysis Systems (SAS Institute; Cary, NC).

RESULTS

Exhaled NO

Exhaled NO in patients with PSS was repeatable (R > 0.9) on the first visit and 4 days later. The values for exhaled NO are shown in Table 1. Exhaled NO was significantly elevated in patients with PSS without ILD compared to healthy control subjects (p < 0.001) and to patients with PSS and ILD (p < 0.001). The DLCO was significantly lower in the patients with PSS and ILD (p < 0.001) but not in PSS without ILD (p > 0.1) compared to healthy control subjects. There was no difference in exhaled NO between healthy control subjects and patients with PSS and ILD (p > 0.4).

There was no bronchial hyperresponsiveness on histamine challenge in patients with PSS with and without ILD (provocative concentration of histamine causing a 20% fall in FEV1 of > 16 mg/mL). There was no difference in exhaled NO in patients with PSS and ILD having esophageal dysmotility (5.9 [0.9] ppb) compared to those without esophageal dysmotility (6.1 [1.0] ppb; p > 0.4).

Exhaled NO After 6 Months of Therapy

There was no difference in exhaled NO between healthy control subjects and patients with PSS and ILD 6 months following therapy with azathioprine and low-dose prednisone (p > 0.8). There was no significant difference in the baseline exhaled NO and

Table 1—Exhaled NO and FEV1 in Healthy Control Subjects and PSS Patients With and Without ILD*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Healthy Control Subjects</th>
<th>Patients With PSS Without ILD</th>
<th>Patients With PSS and ILD (Baseline)</th>
<th>Patients With PSS and ILD Following 6 mo of Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO, ppb</td>
<td>6.3 (0.2)</td>
<td>9.6 (0.7)*</td>
<td>6.2 (0.6)</td>
<td>6.4 (0.3)</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>98 (8)</td>
<td>94 (17)</td>
<td>60 (17)</td>
<td>73 (8)</td>
</tr>
<tr>
<td>DLCO (% predicted)</td>
<td>97 (10)</td>
<td>70 (11)</td>
<td>60 (11)*</td>
<td>68 (11)*</td>
</tr>
</tbody>
</table>

*Data are presented as mean (SEM).
†p < 0.001 t test comparing patients with PSS with healthy control subjects.
‡Measured using the single-breath method.

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exhaled NO following 6 months of therapy in patients with PSS and ILD (p > 0.6). This is shown in Table 1.

**Correlation of Exhaled NO With Other Markers**

There was no correlation of exhaled NO with FEV$_1$ (p > 0.3), FVC (p > 0.4), and DLCO (p > 0.5).

**BALF**

There was a significant difference between healthy control subjects and patients with PSS without ILD in neutrophils (p < 0.001), eosinophils (p < 0.001), and lymphocytes (p < 0.001) in BALF. This is shown in Table 2.

Table 2 also shows a significant difference in the total cell count (p < 0.001), neutrophils (p < 0.001), lymphocytes (p < 0.001), and eosinophils (p < 0.001) in BALF between healthy control subjects and patients with PSS and ILD. There was no significant difference in macrophages (p > 0.4). There was a significant elevation in total cell count, neutrophils, eosinophils, and lymphocytes in patients without ILD and patients with ILD. Exhaled NO did not correlate with total cell counts or individual cell subsets in BALF.

**Discussion**

The present study has shown for the first time (to our knowledge) that exhaled NO is elevated in patients with PSS without clinical or radiologic evidence of ILD. We also demonstrated that exhaled NO is not elevated in patients with PSS and ILD with clinical evidence of pulmonary hypertension. Exhaled NO did not change following 6 months of therapy with azathioprine and low-dose prednisone in patients with PSS and ILD. The BALF in PSS without ILD showed a significantly higher total cell count and significantly higher neutrophils and eosinophils compared to healthy control subjects, confirming subclinical inflammation in patients with PSS.

Exhaled NO was increased in patients with PSS without ILD compared to healthy control subjects and patients with PSS and ILD. The suggestion of subclinical inflammation in this cohort of patients was substantiated by the elevated cell counts on BALF of these patients. A unique observation is the increased exhaled NO in patients with PSS without ILD. This suggests that there is upregulation of NOS and implies subclinical inflammation that is supported by the finding of an abnormal cytology in BALF in this group of patients. This confirms previous observations by Harrison et al., who showed abnormal BALF cytology and pulmonary interstitial inflammation in open-lung biopsy specimens from patients with PSS without clinical ILD. Elevated neutrophils and eosinophils characterized BALF in this condition when compared to healthy control subjects. These findings on BALF are in keeping with the findings of the present study.

This early inflammatory change would most likely upregulate iNOS via the proinflammatory cytokine milieu present in the lung. Exhaled NO may therefore be a sensitive marker of pulmonary inflammation in patients with PSS without ILD. Exhaled NO did not correlate with any cell subset in BALF both in patients with and without ILD. This suggests that exhaled NO measured a different parameter of inflammation compared to the cellularity of BALF.

The exhaled NO in patients with PSS and ILD was no different than in healthy control subjects. A study by Fajac et al. showed that exhaled NO was elevated in patients with PSS and ILD. They did not indicate whether the patients in their study had pulmonary hypertension, and exhaled NO was measured from a pooled sample of exhaled air collected during tidal breathing. Although nose clips were used, contamination may have occurred from the paranasal sinuses because the pharyngeal pressures during tidal breathing are too low to occlude the velum. Khartitonov et al. showed that exhaled NO in patients with PSS and ILD and pulmonary hypertension is lower than healthy control subjects and elevated in patients with ILD without pulmonary hypertension. The authors postulated that endothelial dysfunction im-

**Table 2—Total Cell Count and Differential Cell Count of BALF in Healthy Control Subjects and PSS Patients With and Without ILD***

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total Cells, $\times 10^6$</th>
<th>Macrophages, %</th>
<th>Neutrophils, %</th>
<th>Lymphocytes, %</th>
<th>Eosinophils, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control subjects</td>
<td>4.4 (0.1)</td>
<td>95.8 (0.4)</td>
<td>1.0 (0.1)</td>
<td>2.6 (0.2)</td>
<td>1.0 (0.1)</td>
</tr>
<tr>
<td>PSS and ILD</td>
<td>6.7 (0.4)†</td>
<td>79.6 (3.1)</td>
<td>10.3 (2.1)†</td>
<td>6.3 (2.2)†</td>
<td>4.0 (1.0)†</td>
</tr>
<tr>
<td>PSS without ILD</td>
<td>5.7 (0.2)‡</td>
<td>88.1 (1.1)</td>
<td>5.5 (0.8)‡</td>
<td>4.3 (0.7)‡</td>
<td>2.3 (0.4)‡</td>
</tr>
</tbody>
</table>

*Data are presented as mean (SEM).
†p < 0.001 t test comparing patients with PSS with healthy control subjects.
‡p < 0.001 t test comparing patients with PSS and ILD pretreatment and posttreatment.
paired NO formation in this condition, resulting in a lowered exhaled NO in patients with pulmonary hypertension.

The results of our study are at variance with those of Kharitonov et al.\(^8\) We postulate that this is due to the complex balance of NO production in patients with PSS and ILD. The cytokines interleukin 2, interleukin 5, and tumor necrosis factor are implicated in the alveolitis phase of PSS.\(^15\) These are also the cytokines that upregulate iNOS.\(^5\) The cytokines platelet-derived growth factor and transforming growth factor are implicated in fibroblast proliferation in PSS,\(^16,17\) and are the cytokines that down-regulate iNOS.\(^18\) This is in contrast to PSS without ILD in which the unopposed upregulation of iNOS resulted in elevated exhaled NO.

Another possible reason for differences in exhaled NO between PSS patients with and without ILD is the degree of endothelial injury that occurs between the two groups in this study.\(^19\) Pulmonary fibrosis is invariably associated with vascular injury, causing endothelial dysfunction that decreases NO production in patients with PSS. This was suggested by Kharitonov et al.,\(^8\) who demonstrated low exhaled NO in patients with PSS having pulmonary fibrosis and pulmonary hypertension. In our study, all patients with PSS and ILD had clinical evidence of pulmonary hypertension and a low diffusion coefficient compared to patients with PSS without ILD. This implies a decrease in the vascular bed, endothelial dysfunction, and vascular remodeling resulting in the diminished generation of NO in patients with PSS and ILD. Patients with PSS and no ILD had no evidence of pulmonary hypertension and a normal diffusion coefficient in keeping with normal endothelial function and NO production.

Esophageal dysmotility (on barium swallow) was found in six patients with PSS and ILD and in eight patients with PSS without ILD. Histamine inhalation challenge showed no bronchial hyperresponsiveness. There was no difference in exhaled NO in patients with PSS and ILD with esophageal dysmotility compared to those without esophageal dysmotility. These latter two observations exclude the possible influence of acid aspiration on exhaled NO. Furthermore, PSS patients without ILD and esophageal dysmotility did not demonstrate bronchial hyperresponsiveness. Thus, it is unlikely that reflux esophagitis may explain the elevated exhaled NO in these patients.

There is no difference in exhaled NO between patients with PSS and ILD and healthy control subjects. This may be multifactorial. We postulate that there is no elevation of NO production in areas of fibrosis and the cytokines mediating fibrosis may also inhibit iNOS. Furthermore, oxidants are found in increased concentrations in pulmonary fibrosis. In the background of a raised oxidant concentration, NO would combine preferably with oxidants to form peroxynitrate, thus making less NO available for detection in exhaled air.\(^20,21\) It is unlikely to be entirely due to the use of corticosteroids in these patients since exhaled NO was not elevated before therapy.

Exhaled NO did not change following 6 months of therapy with azathioprine and low-dose prednisone therapy in patients with PSS and ILD. This suggests that therapy may not influence the course of the disease in tissue responsible for NO production in patients with PSS, or the balance of NO production remains static despite these therapeutic modalities.

In summary, we have shown that exhaled NO was not elevated in patients with PSS and ILD and pulmonary hypertension but was significantly higher in patients with PSS without ILD. This may reflect subclinical inflammation in these patients. An abnormality in BALF cytology in patients with PSS without ILD supports this assertion. This suggests that exhaled NO may be a sensitive noninvasive marker of early pulmonary inflammation. Long-term follow-up is needed to ascertain whether exhaled NO and the cellularity of BALF predict the onset of ILD. The patients with PSS without ILD have been followed up for 13 months, and none have developed any evidence of ILD. The NO levels remain elevated. Although these patients are being monitored, it is uncertain how many of these patients will develop ILD. Long-term follow-up of this cohort in our clinic is underway. A larger multicenter study of PSS patients without ILD is necessary to study the significance of the elevated exhaled NO and BALF cytology.

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REFERENCES


7 Fajac I, Kahan A, Menkes CJ, et al. Increased nitric oxide in...
21 Saleh D, Barnes PJ, Giaid A. Increased production of the potent oxidant peroxynitrate in the lungs of patients with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 1997; 155:1763–1769