Exhaled nitric oxide in pulmonary arterial hypertension associated with systemic sclerosis

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Abstract: The fractional exhaled concentration of nitric oxide (FE_{NO}) has been shown to be reduced in idiopathic pulmonary arterial hypertension (PAH) but has not been adequately studied in PAH associated with systemic sclerosis (SSc). We measured FE_{NO} at an expiratory flow rate of 50 mL/s in 21 treatment-naive patients with SSc-associated PAH (SSc-PAH), 94 subjects with SSc without pulmonary involvement, and 84 healthy volunteers. Measurements of FE_{NO} at additional flow rates of 100, 150, and 250 mL/s were obtained to derive the flow-independent nitric oxide exchange parameters of maximal airway flux ($J'aw_{NO}$) and steady-state alveolar concentration (CA_{NO}). FE_{NO} at 50 mL/s was similar ($P = 0.22$) in the SSc-PAH group (19 ± 12 parts per billion [ppb]) compared with the SSc group (17 ± 12 ppb) and healthy control group (21 ± 11 ppb). No change was observed after 4 months of targeted PAH therapy in 14 SSc-PAH group patients ($P = 0.9$). $J'aw_{NO}$ was modestly reduced in SSc group subjects without lung disease (1.2 ± 0.5 nl/s) compared with healthy controls (1.64 ± 0.9; $P < 0.05$) but was similar to that in the SSc-PAH group. CA_{NO} was elevated in individuals with SSc-PAH (4.8 ± 2.6 ppb) compared with controls with SSc (3.3 ± 1.4 ppb) and healthy subjects (2.6 ± 1.5 ppb; $P < 0.001$ for both). However, after adjustment for the diffusing capacity of CO, there was no significant difference in CA_{NO} between individuals with SSc-PAH and controls with SSc. We conclude that FE_{NO} is not useful for the diagnosis of PAH in SSc. Increased alveolar nitric oxide in SSc-PAH likely represents impaired diffusion into pulmonary capillary blood.

Keywords: exhaled nitric oxide, pulmonary arterial hypertension, scleroderma, systemic sclerosis.

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Pulmonary arterial hypertension (PAH) is a leading cause of morbidity and mortality in scleroderma or systemic sclerosis (SSc).[1] Despite therapy with currently available agents, prognosis remains poor with a 3-year survival of 51%–56%.[1-3] The early detection of SSc-associated PAH (SSc-PAH) [4] is critical, because initiating therapy in patients with milder stages of disease may improve long-term outcomes.[5] There is an urgent need to develop reliable and simple biomarkers for the early diagnosis of SSc-PAH and monitoring response to therapy.

A deficiency in nitric oxide (NO) signaling is felt to play an important role in the pathogenesis of PAH,[6] and several studies have described reductions in the fractional NO concentration in exhaled breath (FE_{NO}) in PAH, particularly the idiopathic variety (IPAH), where FE_{NO} has been correlated with hemodynamic severity of disease and prognosis and has been shown to increase in response to therapy.[7] However, there are scarce data on FE_{NO} in SSc-PAH. Earlier studies have been hampered by small patient numbers, variable methodology, and absence of hemodynamic confirmation of PAH.[8-10] Moreover, these studies all used a single expiratory flow rate that cannot distinguish conducting airway versus alveolar source of NO. An increased alveolar concentration of NO has been reported to be of diagnostic value in SSc-associated interstitial lung disease (SSc-ILD) but has not been adequately investigated in SSc-PAH.[11]

We sought to assess FE_{NO} at the conventional expiratory flow rate of 50 mL/s as a potential biomarker in patients with SSc who had confirmed PAH. In addition, we measured FE_{NO} at multiple expiratory flow rates to allow partitioning into conducting airway and alveolar sources.[12] Our hypothesis was that FE_{NO} would be reduced in patients with SSc-PAH compared with patients with SSc without lung disease because of decreased conducting airway NO generation.

METHODS

Subjects

This prospective study was approved by the local institutional review board, and all participants provided written, informed consent. Twenty-four consecutive, treatment-naive patients with SSc-PAH without significant interstitial or obstructive lung disease, as previously described, were enrolled.[13] Fourteen of these patients were evaluated again 4 months after initiation of targeted PAH therapy. A randomly selected cohort of 103 control subjects with SSc without evidence of pulmonary involvement was studied for compari-

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son. The absence of lung disease was defined as (1) no respiratory complaints; (2) pulmonary function testing performed within the previous year showing a forced vital capacity (FVC) and total lung capacity ≥70% of predicted and a forced expiratory volume in 1 s (FEV_1)/FVC ratio ≥0.7; (3) echocardiogram within the past year with normal right heart chamber size and function and estimated right ventricular systolic pressure of <40 mmHg and no evidence of significant left heart disease; (4) if performed, chest radiograph or computed tomography of chest with no significant parenchymal lung disease; and (5) no PAH-specific therapies given within the preceding month, including those used for non-PAH indications. Eighty-four healthy volunteers served as a control group. Exclusion criteria for all groups included smoking within the preceding 6 months, respiratory tract infection within the preceding 2 weeks, history of hyperreactive airways disease, pregnancy, corticosteroid use of >10 mg prednisone daily or equivalent and use of L-arginine, nitrates, or phosphodiesterase inhibitors. Another group of 38 patients with SSc-PAH who were receiving various PAH-specific therapies were also included and provided FE_{NO} only at the expiratory flow rate of 50 mL/s.

FE_{NO} measurements

Online recording of FE_{NO} was performed according to the recommendations of the American Thoracic Society at expiratory flow rates of 50, 100, 150, and 250 mL/s with a chemiluminescent analyzer (NIOX Flex, Aerocrine, Morrisville, NC).[14] For each flow rate, the average of 2 measurements within 10% of each other was taken. To derive the flow-independent NO exchange parameters of maximum airway flux ($J'aw_{NO}$) and steady-state alveolar concentration (CA_{NO}), the 2-compartment model was applied.[12] The Y-intercept and slope of the linear relationship between NO output (FE_{NO} × flow) and flow rate correspond to $J'aw_{NO}$ and CA_{NO}, respectively, after adjustment for the trumpet shape of the airway tree and axial diffusion (model TMAD).[15]

Because CA_{NO} is determined by the uptake of NO by pulmonary capillary blood as well as by production by alveolar wall lining cells,[16] we calculated the diffusing capacity of NO (DL_{NO}) from the measured diffusing capacity of CO (DL_{CO}) on the most recent pulmonary function test, assuming a DL_{NO}/DL_{CO} ratio of 4.3, as described elsewhere.[17] For the healthy controls, a DL_{CO} of 100% predicted (Knudson) was assumed. Subsequently, the alveolar production of NO (VL_{NO}) was derived as the product of CA_{NO} and DL_{NO}.[18]

Statistical analysis

Data are presented as mean ± standard deviation (SD). Comparisons between groups were performed with one-way analysis of variance, followed by Tukey multiple comparison test or Kruskal-Wallis test, followed by Dunn post hoc test, as appropriate. Multivariate linear regression was used to compare CA_{NO} by patient group while adjusting for DL_{CO}. Serial measurements in the SSc-PAH group were analyzed with paired t test. P value <0.05 was considered significant. Analyses were performed with GraphPad Prism (La Jolla, CA) and Stata (College Station, TX).

RESULTS

Subject characteristics

Nine controls with SSc, 3 treatment-naive patients with PAH, and 1 subject with PAH who was receiving treatment were unable to generate an adequate FE_{NO} recording at 50 mL/s. The demographic and clinical characteristics of the remaining 94 controls with SSc, 21 treatment-naive patients with SSc-PAH, 37 patients with SSc-PAH who were receiving therapy, and 84 healthy controls are presented in Table 1. An additional 2 controls with SSc could not generate a reliable FE_{NO} measurement at the highest expiratory flow rate of 250 mL/s, thereby precluding derivation of the flow-independent parameters.

FE_{NO} measurements

Table 2 summarizes the FE_{NO} results at the different expiratory flow rates in the controls with SSc, treatment-naive patients with SSc-PAH, and healthy control groups. There were no significant differences observed for any flow rate. Within the SSc control group, there were no differences between those with limited versus diffuse skin involvement or among patients receiving immunosuppressive therapy versus those who were not receiving such therapy (P values for FE_{NO} at 50 mL/s: 0.49 and 0.98, respectively). FE_{NO} at 50 mL/s in the SSc-PAH patients receiving therapy (17.6 ± 13 parts per billion [ppb]) was comparable to the SSc-PAH treatment-naive group (19.2 ± 12 ppb; P = 0.64). Combining the two SSc-PAH groups, we had >95% power to detect a 33% difference in FE_{NO} at 50 mL/s compared with controls with SSc at a two-sided α of 0.05. Within the SSc-PAH treatment-naive group, there was no relationship between FE_{NO} at 50 mL/s and mean pulmonary artery pressure or pulmonary vascular resistance index (data not shown).

Two-compartment model and VL_{NO} calculation

Maximal airway flux of NO ($J'aw_{NO}$) in patients with SSc without pulmonary involvement was similar to that in subjects with SSc-PAH but significantly less than that in healthy volunteers (Fig. 1). In contrast, CA_{NO} was considerably increased in the SSc-PAH group compared with both the SSc control group and healthy volunteers (Fig. 2*A*). CA_{NO} was also significantly higher in patients with SSc without pulmonary involvement than in healthy controls. However, after adjusting for DL_{CO}, there was no longer a significant difference in CA_{NO} between the SSc control group and SSc-PAH group (P = 0.08). The alveolar production of NO (VL_{NO}) calculation also showed no difference between controls with SSc and the SSc-PAH group. Both SSc groups had reduced VL_{NO} values compared with healthy controls (Fig. 2*B*).

Changes in response to specific PAH therapy

As shown in Figure 3, no change was noted in FE_{NO} at 50 mL/s (P = 0.9) in 14 patients with SSc-PAH after 4 months of treatment. Specific PAH therapies were a combination of tadalafil and ambrisentan as part of an open-label clinical trial[13] (N = 5) and monotherapy with tadalafil (3), sildenafil (3), ambrisentan (1), bosentan (1), and inhaled treprostinil (1). Similar findings were observed for

Table 1. Demographic and clinical characteristics

Variable	SSc controls (N = 94)	Patients with SSc-PAH (N = 21)	Patients with SSc-PAH receiving therapy (N = 37)	Healthy controls (N = 84)
Age, years	52 (10)	66 (10)	63 (12)	50 (13)
Sex, F/M	87/7	20/1	29/8	43/41
Race, white/black/other	86/5/3	18/2/1	29/8/0	58/15/11
Height, cm	166 (9)	160 (6)	165 (9)	169 (10)
Skin involvement, limited/diffuse	53/41	17/4	30/7	. . .
Immunosuppressive therapy, %	30	14	16	. . .
Six-minute walk distance, m	. . .	292 (158)	335 (128)	. . .
Pulmonary function:				
FVC, % predicted	97 (14)	88 (13)	73 (19)	. . .
FEV_1/FVC ratio	0.79 (0.05)	0.77 (0.05)	0.75 (0.09)	. . .
TLC, % predicted	98 (15)	91 (11)	82 (20)	. . .
DL_{CO}, % predicted	90 (19)	58 (17)	47 (17)	. . .
Hemodynamic characteristic				
Mean PAP, mmHg	. . .	38 (13)	40 (14)	. . .
RAP, mmHg	. . .	7 (4)	9 (5)	. . .
PAWP, mmHg	. . .	11 (4)	12 (4)	. . .
Cardiac index, L/min/m^2	. . .	2.7 (0.9)	2.6 (0.6)	. . .

Note: Data are presented as mean value (± standard deviation), unless otherwise indicated. DL_{CO}: diffusing capacity of CO; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; PAH: pulmonary arterial hypertension; PAP: pulmonary artery pressure; PAWP: pulmonary artery wedge pressure; RAP: right atrial pressure; SSc: systemic sclerosis; TLC: total lung capacity.

$J'aw_{NO}$ and C_ANO (data not shown). As a group, this small cohort did not demonstrate a significant clinical response to therapy in terms of 6-minute walk distance.

DISCUSSION

The main findings of this study are (1) FE_{NO} at 50 mL/s in patients with SSc-PAH is similar to that in subjects with SSc without lung involvement and healthy control subjects; (2) $J'aw_{NO}$ is modestly lower in individuals with SSc without lung disease relative to healthy controls but is similar to that in individuals with SSc-PAH; (3) unadjusted steady-state alveolar concentration (CA_{NO}) is higher in individuals with SSc-PAH than in those with SSc without pulmonary involvement but not after correcting for DL_{CO}, and calculated alveolar production of NO (VL_{NO}) is similar; and (4) no change in any exhaled NO variable was observed in subjects with SSc-PAH after 4 months of specific PAH therapy.

These observations are in contrast to those reported in IPAH. Lower airway NO sampled bronchoscopically as well as NO oxidative reaction products were found to be decreased in patients with

Table 2. Fractional exhaled concentration of nitric oxide (FE_{NO}) measurements

Variable	SSc controls (N = 94)[a]	SSc-PAH (N = 21)	Healthy controls (N = 84)	P value
FE_{NO} 50 mL/s, ppb	17 (6)	19 (12)	21 (11)	0.22
FE_{NO} 100 mL/s, ppb	11 (4)	13 (6)	13 (6)	0.53
FE_{NO} 150 mL/s, ppb	8 (3)	10 (5)	9 (4)	0.58
FE_{NO} 250 mL/s, ppb	6 (2)	7 (4)	6 (3)	0.41

Note: PAH: pulmonary arterial hypertension; ppb: parts per billion.

[a] Two control subjects with systemic sclerosis (SSc) could not generate an adequate FE_{NO} recording at the highest flow rate of 250 mL/s.

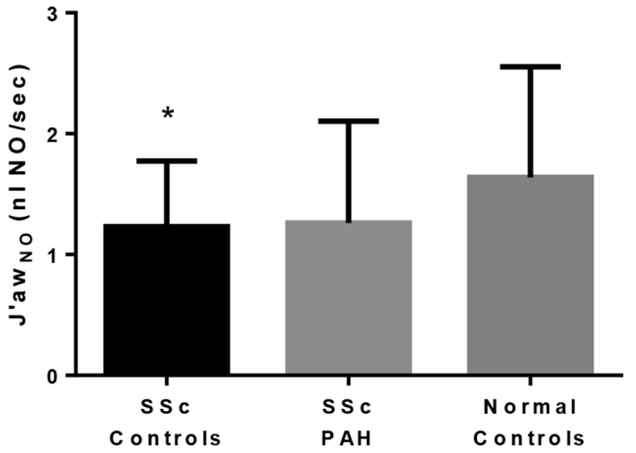

Figure 1. Maximal airway flux of nitric oxide ($J'aw_{NO}$) in patients with systemic sclerosis (SSc) without lung involvement (N = 92), treatment-naive subjects with SSc and pulmonary arterial hypertension (PAH; N = 21), and healthy control subjects (N = 84). Error bars represent standard deviation. Single asterisk indicates $P < 0.05$ compared with healthy controls.

IPAH relative to healthy controls.[19] Our group has also shown a reduction in FE_{NO} at 50 mL/s in this population,[20] along with a low airway wall concentration of NO using the 3-compartment model.[12] Moreover, these abnormalities reversed after 3 months of therapy with the dual endothelin-receptor antagonist bosentan. In a mixed population of individuals with PAH (mainly IPAH), Machado et al.[21] found an inverse correlation between FE_{NO} and pulmonary artery pressure and better survival among those with an increase in FE_{NO} on serial testing.

Our results indicate that FE_{NO} is not a useful diagnostic biomarker for PAH in patients with SSc. Despite the relatively small sample size, we had adequate power to demonstrate a biologically or clinically meaningful difference between SSc subjects without pulmonary involvement and SSc-PAH, particularly when combined with a group of patients receiving specific PAH therapy. Combining these 2 groups was justified, because their FE_{NO} values were similar, and the latter did not change in response to therapy in treatment-naive patients with SSc-PAH. As expected, there was considerable individual variability among all groups, but our sample size precluded the ability to investigate any potential association between FE_{NO}, severity of disease, prognosis, or response to therapy.

Earlier studies of exhaled NO in SSc-PAH were limited by variable methodology, small sample size, and inadequate patient characterization. Kharitonov and coworkers[8] and Rolla and colleagues[10] reported significantly reduced FE_{NO} compared with subjects with SSc without lung disease. However, expiratory flow rate, which dramatically influences FE_{NO},[14] was not adequately controlled. Malerba et al.[9] assessed FE_{NO} at 50 mL/s and found significantly reduced values among patients with SSc with ILD alone or isolated PAH compared with those without lung involvement, roughly 11 versus 20 ppb. Those with combined ILD and PH had the lowest values, ~6 ppb. Both this study and that of Rolla's described an inverse correlation between FE_{NO} and Doppler estimated pulmonary artery systolic pressure.[9,10] All 3 studies lacked hemodynamic confirmation of PH by right heart catheterization.

The basis for the absence of reduced FE_{NO} in SSc-PAH compared with our previous findings in treatment-naive IPAH is not clear.[20] Although the hemodynamic severity of disease was less in the current study, as is typical for SSc compared with IPAH,[22] there was no suggestion that FE_{NO} was lower in those with higher pulmonary artery pressure or vascular resistance. Immune activation leads to increased expression of inducible NO synthase (iNOS) in a variety of cell types,[23,24] and circulating NO metabolites are consistently elevated in SSc,[25,26] even among subjects with pulmonary hypertension.[27,28] Thus, inflammatory mechanisms may overshadow reduced NO production by the constitutive endothelial and neuronal NOS isoforms.[24,29]

Our finding of a modestly lower $J'aw_{NO}$ in patients with SSc without pulmonary involvement relative to healthy controls mirrors earlier results of a study involving subjects with SSc-ILD.[17] The dif-

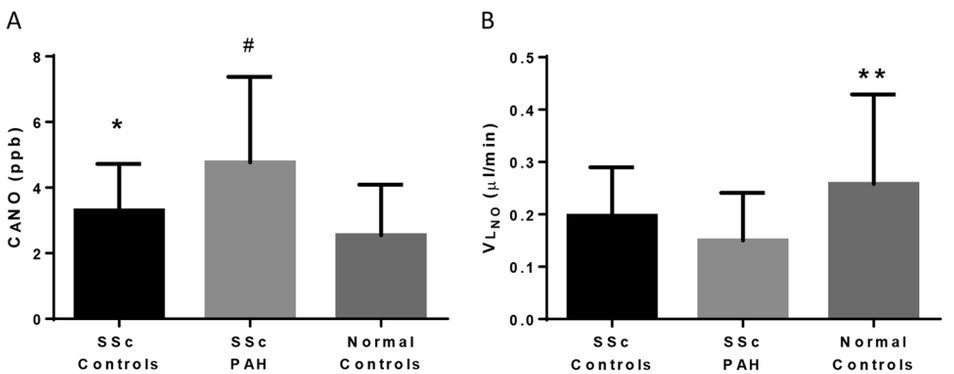

Figure 2. Steady-state alveolar concentration of nitric oxide (CA_{NO}; A) and calculated alveolar production of nitric oxide (VL_{NO}; B) in patients with systemic sclerosis (SSc) without lung involvement (N = 92), treatment-naive subjects with SSc and pulmonary arterial hypertension (PAH; N = 21), and healthy control subjects (N = 84). Error bars represent standard deviation. One asterisk indicates $P < 0.01$ compared with healthy controls. Number sign indicates $P < 0.05$ compared with controls with SSc. Two asterisks indicate $P < 0.05$ versus controls with SSc and the SSc-PAH group.

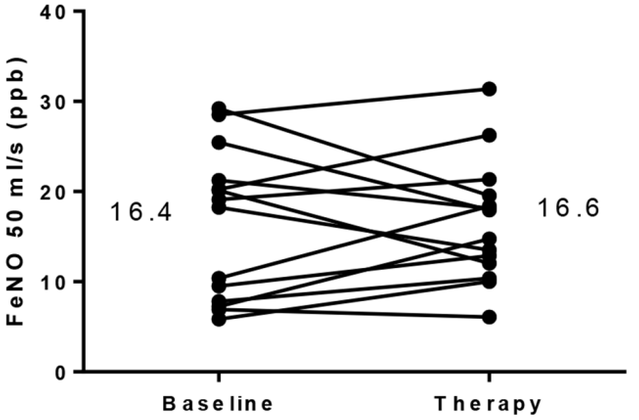

Figure 3. The fractional exhaled concentration of nitric oxide (FE_{NO}) at 50 mL/s in parts per billion (ppb) at baseline and after 4 months of targeted pulmonary arterial hypertension (PAH) therapy in 14 patients with systemic sclerosis and PAH. Mean values at each time point are shown.

ference was larger in the latter study, which found a mean value in patients with SSc-ILD that was half that observed in healthy subjects. Tiev et al.[30] also found significantly lower $J'aw_{NO}$ in SSc-ILD compared with SSc without ILD, although others reported normal values.[31] The determinants of maximal bronchial NO flux include its production by airway wall relative to catabolism.[16] The presence of reactive oxygen species, which play a central role in the pathogenesis of systemic sclerosis,[32] would enhance the degradation of NO and could contribute to reduced $J'aw_{NO}$.

Elevated alveolar concentrations of NO have been a consistent finding in SSc.[17,30,31] Tiev et al.[30] reported median CA_{NO} values of 7.5, 4.9, and 2.0 ppb in SSc-ILD, SSc without ILD, and healthy controls, respectively. These investigators also showed an association with subsequent decrease in pulmonary function,[33] the presence of alveolitis defined by bronchoalveolar lavage fluid cellular differential,[34] and the clinical response to cyclophosphamide therapy,[35] suggesting that CA_{NO} reflects alveolar inflammation. Wuttge et al.[31] found a weak correlation between CA_{NO} and the extent of ground-glass opacities and reticulations on high-resolution chest CT but not with pulmonary function variables.

Steady-state alveolar NO concentration reflects the balance between production by alveolar lining cells (VL_{NO}) and the diffusion capacity into pulmonary capillary blood (DL_{NO}). Because the avidity of hemoglobin for NO is extremely high, DL_{NO} is largely determined by the membrane component of gas transfer rather than pulmonary capillary blood volume.[36] In the absence of a direct measurement, we calculated DL_{NO} using an assumed DL_{NO}/DL_{CO} ratio. The average ratio of 4.3, derived from healthy volunteers,[36] closely approximates the value of 4.41 in a group of patients with SSc without lung disease and 4.29 in a small number of subjects with SSc-PAH.[37] In this study, although CA_{NO} in the SSc-PAH group was significantly higher than in both the SSc group and healthy controls, the VL_{NO} calculation did not suggest increased alveolar NO production relative to either group. These findings are similar to an earlier report involving SSc-ILD.[17] The basis for the statistically lower VL_{NO} in the SSc group compared with the healthy control group is unclear, but it could be partly explained by a higher proportion of male subjects and younger mean age in the latter, with a consequently higher predicted DL_{NO}. The alveolar compartment in the model includes the most distal airways,[38] where subtle abnormalities are common in SSc,[39,40] which could contribute to reduced NO production.[17] Enhanced oxidative catabolism, as invoked for the reduced bronchial flux, may also be a factor. The loss of a significant difference in CA_{NO} between the SSc-PAH group and the control group with SSc after adjusting for DL_{CO} provides additional support for the absence of increased NO production in the alveolar compartment.

In summary, we have demonstrated that FE_{NO} in patients with SSc-PAH is similar to that in subjects with SSc without pulmonary involvement and healthy controls. Furthermore, we found no change in FE_{NO} after 4 months of specific PAH therapy. Elucidation of the basis for and significance of the observed elevation in alveolar NO concentrations in SSc lung disease will require concomitant measurements of DL_{NO}. Additional studies including direct comparison with IPAH are needed to clarify differences between these subtypes.

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Conflict of Interest: SCM serves as a consultant for Actelion, Bayer, and United Therapeutics; receives research funding from NIH/NHLBI and the Scleroderma Research Foundation; and is on the speakers bureau for the Pulmonary Hypertension Association. PMH is on the advisory board for scientific affairs of Gilead Sciences. REG serves on the speakers bureau of Gilead Sciences and Bayer Pharmaceuticals and participates in clinical research trials sponsored by Pfizer and United Therapeutics. All other authors: none declared.

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