



# Effect of inhaled nitric oxide on pulmonary function in cystic fibrosis

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Concentrations of nitric oxide (NO) have been found to be reduced in both the upper and lower airway of patients with cystic fibrosis (CF). As NO modulates bronchomuscular tone, low NO levels may contribute to the obstructive lung disease in these patients. To assess whether increasing inspiratory NO concentrations has any impact on lung function, we have studied 13 CF patients aged 14–38 years in a clinically stable condition and nine healthy controls. NO was applied via a mixing chamber for 5 min with NO concentrations of 100 parts per billion, 1 and 40 parts per million. Spirometry was performed at baseline and after inhalation on each occasion.

There were no clinical side-effects at any NO concentration and no changes in oxygen saturation were observed. Lung function remained unchanged in all subjects throughout the study period. Sputum nitrate and nitrite concentrations before and after inhalation of high NO concentrations (40 ppm) in eight CF patients did not show any significant changes, even though a tendency to higher nitrate levels was observed ( $399 \pm 231$  vs.  $556 \pm 474 \mu\text{mol l}^{-1}$ ). Therefore, inhaled NO at either the physiological levels present in the upper airway of normal individuals or those used therapeutically to treat pulmonary hypertension has no immediate effect on bronchomuscular tone in patients with cystic fibrosis.

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## Introduction

Lung disease in cystic fibrosis (CF) is characterized by chronic, neutrophil dominated airway inflammation (1). Despite high levels of pro-inflammatory cytokines, which are known to induce enzymes that produce nitric oxide (NO), recent studies have shown that NO concentrations in both upper and lower airways of CF patients are decreased (2,3). This may be due to reduced expression of the inducible NO synthase (NOS2) in CF epithelial cells, as recently reported (4,5). In addition, there is evidence for impaired function of bronchodilatory nerve fibres in CF which use NO as a neurotransmitter (6).

The pathophysiological significance of reduced NO formation in CF airways remains unclear. NO has a number of functions that may be relevant for CF lung disease. NO stimulates ciliary beating frequency (7) and is involved in neutrophil-mediated killing of *Staphylococcus aureus*, a common pathogen in CF patients (8–10). In addition, NO is a potent vasodilator (11,12) and acts as a bronchodilator as shown by a reduced bronchoconstrictor response to metacholine after pre-treatment with NO (13).

NO levels in the nose are 100-fold higher than in the lower airways and it has been postulated that auto-

inhalation of upper airway NO is important in maintaining bronchomuscular tone (14). Therefore, reduced concentrations of NO in the upper airway could potentially have a negative impact on the obstructive lung disease in CF. In this study, we assessed whether inhalation of NO in concentrations present in the upper airway of normal individuals as well as concentrations used therapeutically in diseases such as pulmonary hypertension have any impact on pulmonary function in CF patients.

## Material and Methods

Thirteen CF patients (median age: 16.5 years, range: 14–39) and nine non-smoking healthy controls (median age 26.5 years, range 24–37) were included in this study. The diagnosis of cystic fibrosis had been confirmed by repeated sweat test with chloride levels exceeding  $60 \text{ mval l}^{-1}$ . All patients were clinically stable at the time of study. None of the patients received corticosteroids; bronchodilators were withheld for at least 8 h prior to study. The controls were free of acute or chronic respiratory disease and had no history of or clinical evidence for hyperreactive airway disease. None of the controls received any medication. The study was approved by the ethics committee of our institution; written informed consent was obtained by all volunteers and patients or their parents.

NO was applied via a mixing chamber (Loflo, Järfälla, Sweden) which allowed supplementing NO to synthetic air containing 21% oxygen over a wide range of NO concentrations. The output of the mixing chamber was

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connected to a mouth-piece via a three-way valve and to a 3-l anaesthetic bag. In preliminary experiments we could demonstrate constant inspiratory NO as well as oxygen concentrations with this setting. The concentration of NO during inhalation was monitored continuously via a chemiluminescence analyser (NO 280, Sievers, Boulder, CA, U.S.A.). Subjects were instructed to perform normal tidal breathing over a period of 5 min. NO inhalations were performed with concentrations of 100 ppb, 1 ppm and 40 ppm, respectively, on 3 consecutive days in random order.

Spirometry was performed at baseline on each day and immediately after inhalation of the NO gas mixture. Three forced expiratory manoeuvres from total lung capacity were performed on each occasion; the mean of the three measurements was taken for analysis. The primary outcome parameter was a change in forced expiratory volume in 1 sec (FEV<sub>1</sub>) from baseline, but changes for forced vital capacity (FVC) and flows at lower lung volumes mid-expiratory flow [(MEF) 50 and 25% of VC] were also included in the analysis. Oxygen saturation was monitored continuously during the experiments; blood pressure was measured before and after each experiment.

To assess the amount of NO retained as stable degradation products in respiratory secretions, sputum was sampled before and after inhalation of 40 ppm NO. Sputum samples were diluted 1:1 in aqua bidest and centrifuged at 200 g for 10 min after being homogenized in an ultrasound water bath as previously described (15). Clear supernatant was used for analysis. Nitrate was reduced to NO with vanadium III chloride (4 ml of a solution containing 800 mg vanadium III chloride, 8 ml of a 37% solution of HCL and 100 ml of distilled water). The evolved NO was determined with a Sievers NO analyser by

comparing results against a standard curve generated before each experiment. Similarly, nitrite was measured by reduction with 3 ml of acetic acid and 1 ml of a 5% solution of potassium iodide. Each test was performed in triplicate; the mean of three measurements was used for analysis.

## STATISTICAL ANALYSIS

All data were tested for normal distribution with the Kolmogorov-Smirnov test. Results were expressed as mean  $\pm$  standard deviation (SD). The differences in pulmonary function and nitrate concentration in sputum before and after NO inhalation was assessed with the paired Student's *t*-test. Linear regression analysis was used for the comparison of NO concentration in exhaled air and nitrate concentration in sputum. Changes in nitrate and nitrite concentration after incubation with NO were tested with analysis of variance (ANOVA). A *P*-value of less than 0.05 was considered statistically significant.

## Results

NO inhalation was well tolerated in all CF patients and control subjects. Oxygen saturation, heart rate and blood pressure remained unchanged during the inhalation period at all NO concentrations.

The effect of NO inhalation in concentrations of 100 ppb, 1 ppm and 40 ppm on pulmonary function in normal subjects and CF patients is displayed in Table 1. There were no changes in FEV<sub>1</sub>, FVC or flows at lower lung volumes for all NO concentrations in both healthy individuals and patients with CF. Individual response did not differ from the group overall and none of the subjects had a change in

TABLE 1. Pulmonary function in cystic fibrosis (CF) patients and controls receiving different concentrations of inhaled nitric oxide

	100 ppb		1 ppm		40 ppm	
	Pre	Post	Pre	Post	Pre	Post
<b>Controls</b>						
FVC (% predicted)	102.8 $\pm$ 8.6	102.2 $\pm$ 7.8	101.3 $\pm$ 8.9	103.2 $\pm$ 8.9	105.9 $\pm$ 9.0	104.4 $\pm$ 8.7
FEV <sub>1</sub>	104.6 $\pm$ 9.3	104.2 $\pm$ 10.1	103.6 $\pm$ 10.4	103.6 $\pm$ 10.4	106.5 $\pm$ 9.9	105.0 $\pm$ 10.5
PEFR	101.9 $\pm$ 21.0	96.5 $\pm$ 18.8	103.0 $\pm$ 19.5	103.0 $\pm$ 19.5	105.9 $\pm$ 19.7	103.9 $\pm$ 20.4
MEF 50% VC	97.6 $\pm$ 21.3	97.7 $\pm$ 3.3	97.7 $\pm$ 20.1	94.9 $\pm$ 21.0	101.9 $\pm$ 15.8	97.0 $\pm$ 15.6
MEF 25% VC	92.9 $\pm$ 19.7	92.5 $\pm$ 27.5	90.0 $\pm$ 20.2	91.9 $\pm$ 21.1	93.4 $\pm$ 12.3	89.9 $\pm$ 22.8
<b>Cystic fibrosis</b>						
FVC (% predicted)	68.1 $\pm$ 21.9	69.5 $\pm$ 23.8	67.5 $\pm$ 17.9	66.4 $\pm$ 19.7	71.7 $\pm$ 21.3	73.3 $\pm$ 21.5
FEV <sub>1</sub>	58.0 $\pm$ 26.6	59.5 $\pm$ 27.4	58.2 $\pm$ 21.3	58.2 $\pm$ 21.9	60.8 $\pm$ 23.7	61.5 $\pm$ 23.7
PEFR	63.6 $\pm$ 22.5	64.6 $\pm$ 22.5	67.5 $\pm$ 28.4	65.9 $\pm$ 26.1	68.4 $\pm$ 22.0	67.8 $\pm$ 26.2
MEF 50 % VC	44.9 $\pm$ 43.3	45.2 $\pm$ 41.2	44.5 $\pm$ 35.3	42.5 $\pm$ 33.7	40.1 $\pm$ 28.1	40.4 $\pm$ 31.9
MEF 25% VC	33.0 $\pm$ 31.6	30.5 $\pm$ 24.4	28.6 $\pm$ 21.0	30.9 $\pm$ 21.2	26.6 $\pm$ 28.4	30.4 $\pm$ 31.5

ppb, parts per billion; ppm, parts per million.  
Abbreviations as in text.

any of the lung function parameters exceeding 10% of the baseline value.

Sputum nitrate concentrations for eight patients who produced sufficient sputum material at baseline were similar to our results reported previously (15). There was a significant correlation between exhaled NO concentrations and sputum nitrate ( $r = 0.83$ ,  $P < 0.02$ , linear regression analysis) (Fig. 1).

Sputum nitrate and nitrite concentrations before and after inhalation of high NO concentrations (40 ppm) did not show any significant changes, even though a tendency to higher nitrate levels was observed ( $399 \pm 231$  vs.  $556 \pm 474 \mu\text{mol l}^{-1}$ ).

To study the dynamics of metabolism of NO to its stable degradation products nitrate and nitrite, we incubated sputum of five CF patients with room air containing 40 ppm NO over a period of 5 min and measured nitrate and nitrite concentrations immediately after incubation, after 1, 3, 6 and 24 h. Compared to untreated specimens of the same individuals, nitrate concentrations remained unchanged over the observation period, a significant increase was observed in nitrite levels after 1 h of incubation ( $P < 0.05$ , ANOVA) which remained significant up to 6 h (Fig. 2).

### Discussion

In a previous study we showed that L-arginine, the substrate of NO synthases, given intravenously in a dose of  $500 \text{ mg kg}^{-1}$  bodyweight, results in a small but significant increase in lower airway NO formation as reflected by increased NO concentrations in end-tidal exhaled air (16). However, even with these high doses of L-arginine, normalization of airway NO was not achieved and lung function remained unchanged. In the present study we have, therefore, tested whether inhaled NO has any bronchodilator effect in CF patients. The results of the present study demonstrate that neither the concentrations present in the upper airway of normal individuals or higher concentrations (40 ppm) that are used therapeutically for

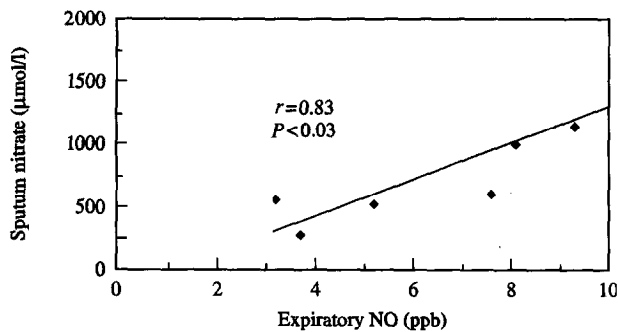


FIG. 1. Exhaled NO concentrations and nitrate concentrations of sputum in seven CF patients. Measurements were obtained at baseline before the inhalation of NO. Each point represents one individual. A positive correlation was observed between NO in exhaled air and its stable degradation product nitrate in sputum ( $P = 0.02$ ,  $r = 0.82$ , linear regression analysis).

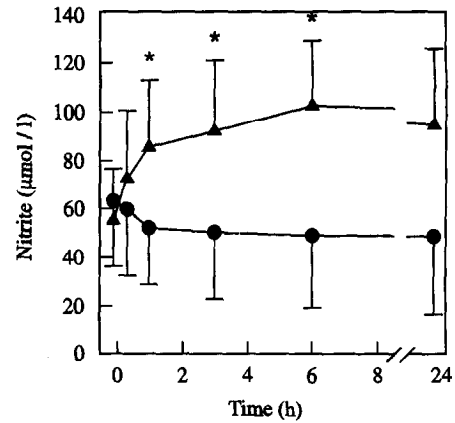


FIG. 2. Changes in nitrite concentrations in sputum after incubation with 40 ppm NO (▲) compared to untreated sputum specimens (●). The mean and SEM are displayed before incubation (0), after 10 minutes, 1, 3, 6 and 24 h. A significant increase was observed after 1 h which remained elevated up to 6 h ( $*P < 0.05$ ; ANOVA).

pulmonary hypertension have any effect on pulmonary function in patients with cystic fibrosis. Therefore, the low levels of NO in airways of CF patients appear to be of no relevance for the control of bronchomuscular tone in CF patients

The effect of inhaled NO on bronchomuscular tone has been studied before in animal models with metacholine-induced bronchoconstriction and in adult subjects with and without hyperreactive airway disease (13,17,18). While inhaled NO in a dose of 80 ppm had no effect on metacholine-induced bronchoconstriction in normal individuals, a bronchodilatory effect was seen in subjects with hyperreactive airways and/or asthma (13). This effect was, however, less pronounced than that achieved by  $\beta_2$ -agonists. While we could confirm previous findings that NO has no effect on resting bronchomuscular tone in normal individuals, this also appears to be the case in cystic fibrosis, where NO formation is reduced.

Due to the secretion barrier, inhaled NO may not reach the bronchial smooth muscle cells in CF. This is, however, unlikely as inhaled NO used therapeutically for pulmonary hypertension can easily cross the alveolar membrane and reach the muscular layer of arterioles (19). In addition, protection against histamine-induced bronchoconstriction has been described in asthmatic subjects who also have an increased diffusion barrier due to airway oedema (13). While retention of NO within respiratory secretions may limit the concentrations in the lower airways in CF, our data on NO metabolites in sputum would not support that this deposition of NO within the airway secretion alone can explain the lack of effect of inhaled NO on pulmonary function in CF patients.

We have chosen a maximal NO concentration of 40 ppm which is known to be effective in lowering pulmonary vascular tone and is also reported to be safe (20). In the presence of oxygen, NO is metabolized to  $\text{NO}_2$  which is a toxic gas at concentrations exceeding 5 ppm. Based on theoretical calculations, this concentration is reached within

13 min at 21% oxygen and 40 ppm, but in only 3 min, if 80 ppm of NO are applied (21,22). In addition, other potentially harmful toxic metabolites such as nitrous acid, nitric acid and peroxynitrite may be formed and retained in respiratory secretion (23). Although we cannot exclude the possibility that NO concentrations higher than 40 ppm may have some effect on bronchomuscular tone, we would not test their efficacy unless their safety has been clearly demonstrated.

The time-course of oxidation of NO to its stable metabolites, nitrate and nitrite, within CF airways has not been defined. Dynamics of increase in sputum NO metabolites are not only influenced by the concentrations of NO and oxygen but may also be altered by respiratory diseases due to the presence of sputum. Sputum has recently been shown to have a rather low oxygen tension (24). Although we have previously found elevated concentrations of nitrate and nitrite in sputum of CF patients (15), this metabolization may take longer than that chosen for sputum collection in the present study (10 min). This is supported by our *in vitro* measurements, where nitrite concentrations were unchanged immediately after incubation with 40 ppm NO but increased significantly after 1 h.

In addition to its effect on airway and vascular smooth muscle cells, NO has multiple functions which are potentially relevant for CF lung disease. Nitric oxide stimulates ciliary beating frequency and low NO levels in the airway could thus impair mucociliary clearance (7). NO has antimicrobial activities and is needed for neutrophil-mediated killing of *S. aureus*, a common pathogen in CF lung disease (9,25). Finally, NO has been shown to activate CFTR, thereby increasing chloride current through epithelial cells (26,27). The relevance of these functions for patients with cystic fibrosis has not been defined. While our data show that the increasing NO in inspired air has no immediate effect on lung function of CF patients, other aspects mediated by NO may still be functionally relevant for CF lung disease.

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