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SHORT REPORT

Glucocorticoid treatment reduces exhaled nitric oxide in cystic fibrosis patients

S.J. Linnane*, A.G. Thin#, V.M. Keatings*, J.B. Moynihan#, P. McLoughlin#, M.X. FitzGerald*

Glucocorticoid treatment reduces exhaled nitric oxide in cystic fibrosis patients. S.J. Linnane, A.G. Thin, V.M. Keatings, J.B. Moynihan, P. McLoughlin, M.X. FitzGerald. ©ERS Journals Ltd 2001.

ABSTRACT: In cystic fibrosis (CF), low concentrations of exhaled nitric oxide (NO) and reduced expression of inducible nitric oxide synthase (iNOS) in airway epithelium have been reported. However, abundant iNOS expression has been found in the subepithelial tissues and elevated concentrations of NO metabolites in breath condensate and sputum. These conflicting results may be explained by increased scavenging of NO by superoxide radicals, resulting in rapid conversion to peroxynitrite, so that only a small proportion of the NO produced in the lung tissue reaches the airway lumen. If iNOS were active in the CF lung, exhaled NO would be further reduced by glucocorticoid treatment.

CF patients (n = 13) were recruited to a double-blind, placebo-controlled study with crossover. Treatment comprised prednisolone or placebo for 5 days with a 9 day washout. After each treatment, exhaled NO was measured, spirometry performed and blood collected for measurement of serum nitrogen dioxide/nitrous oxide (NO_2/NO_3).

Ten patients (8 male) completed the study. Following prednisolone treatment (mean ± SD) exhaled NO concentration (3.1 ± 1.6 parts per billion (ppb)) was significantly reduced versus placebo treatment (4.9 ± 4.2 ppb; $p < 0.05$, Wilcoxon signed-rank test). Spirometric indices and serum NO_2/NO_3 concentration were unchanged.

These findings support the hypothesis that glucocorticoids suppress nitric oxide production in cystic fibrosis airways by reducing inducible nitric oxide synthase expression or by inhibiting recruitment of neutrophils, cells which express inducible nitric oxide synthase.

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*Dept of Medicine and Therapeutics and #Dept of Physiology, Conway Institute of Biomolecular Medicine, University College Dublin, Dublin, Ireland.

Correspondence: P. McLoughlin, Dept of Physiology, Conway Institute of Biomolecular Medicine, University College Dublin, Earlsfort Terrace, Dublin 2, Ireland.
Fax: 35317067417

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Low concentrations of nitric oxide (NO) in expired airway gases of cystic fibrosis (CF) patients [1–3] and reduced expression of inducible nitric oxide synthase (iNOS) in CF airway epithelium has been reported [4, 5]. However, abundant iNOS expression has been found in the subepithelial tissues of CF lungs [5] and an elevated concentration of NO metabolites has been reported in breath condensate [6] and in sputum [2, 7]. A potential explanation of these conflicting results is that there is increased scavenging of NO by superoxide radicals in the inflamed CF lung [8] resulting in the rapid conversion of NO to peroxynitrite, so that only a small proportion of the NO produced in lung tissue reaches the airway lumen. Peroxynitrite is a highly reactive molecule, which has been shown to cause tissue damage by a number of mechanisms [9]. iNOS expression is reduced by glucocorticoids but expression of endothelial and neuronal NOS is not [10]. Short courses of both oral and inhaled glucocorticoids have been shown to reduce both iNOS expression and exhaled NO [11–14]. Therefore, if iNOS contributes to NO production in CF lungs, glucocorticoid treatment would be expected to reduce exhaled NO. The purpose of this study was therefore to measure the effect of oral glucocorticoid treatment on exhaled NO in patients with CF.

Methods

Patients

Thirteen patients with documented CF on the basis of clinical history and abnormal sweat electrolyte measurements (chloride > 60 mmol·L^{-1}) were recruited from the National Adult Cystic Fibrosis Unit, St. Vincent's University Hospital (Dublin, Ireland, UK). The study protocol was approved by the Ethics Committee of St. Vincent's University Hospital and all patients gave written informed consent. All patients were free of acute pulmonary exacerbation of CF for at least 2 months. Patients were excluded if they had a history of glucose intolerance, diabetes mellitus, major recent haemoptysis or pneumothorax. All patients had positive sputum cultures for *Pseudomonas aeruginosa*. No patients were taking oral glucocorticoids, nonsteroidal anti-inflammatory medication or any vasoactive

medication at the time of recruitment. Three patients were on inhaled glucocorticoids (Becotide Inhaler® 500 μg, twice a day (two patients) and Flixotide Inhaler® 500 μg, twice a day (one patient)) for at least 1 yr prior to the study.

Study design

Patients withheld inhaled glucocorticoids during a 2-week run-in phase and throughout the study. Treatment was assigned in a double-blinded manner with patients receiving either prednisolone ($0.5\ mg \cdot kg^{-1}$ rounded up to the nearest 5 mg) or matched placebo, each morning for 5 days. On the fifth treatment day they were reviewed, spirometry performed, Schwachman scores calculated, exhaled NO concentration measured and blood collected for full and differential cell counts and the measurement of serum nitrite/nitrate oxide (NO_2/NO_3) and C-reactive protein (CRP). A 9-day washout phase then preceded the second 5-day period of treatment with prednisolone or placebo after which the patients were again reviewed.

Measurements

Exhaled NO concentration was measured using a chemiluminescent analyser (LR 2000, Logan Research Ltd, Rochester, UK) and conformed to American Thoracic Society guidelines [15]. The analyser was calibrated regularly using standard calibration gases (BOC gases Ltd, Dublin, Ireland) and ambient NO concentration was always <4 parts per billion (ppb). Patients exhaled against a fixed resistance while maintaining mouth pressure at 8–10 cmH_2O guided by a visual display of mouth pressure giving a flow rate $\sim 0.43\ L \cdot s^{-1}$. Lower airway NO concentration was determined at the end of exhalation, as indicated by the plateau phase of the simultaneously measured carbon dioxide (CO_2) concentration. This protocol ensured all measurements were made at constant expiratory flow rate and thus any changes directly reflected changes in airway NO excretion. The standard deviation (SD) of repeated measurements was 1.2 ppb. The mean of three measurements was recorded for each subject on each occasion. Spirometry was performed using a Vmax229 (Sensor Medics, Yorba Linda, CA, USA). Serum NO_2/NO_3 concentration was measured using the Griess reaction as previously described [16].

Statistical analysis

All data are expressed as mean±SD. Wilcoxon signed-rank test was used to assess differences between glucocorticoid and placebo treatments.

Results

Thirteen patients (eight male) were recruited to the study, three of these patients did not complete the study because of intercurrent illness and were excluded from analysis. Percentage predicted forced expiratory volume in one second (FEV1% pred) for these three were within the range of the other 10. Anthropometric data (mean±SD) for the 10 (eight male) patients who completed the study protocol were as follows: age 21.1±2.0 yrs, height 1.75±0.09 m, weight 65.5±12.6 kg, body mass index $21.2 \pm 2.5\ kg \cdot m^{-2}$, Schwachman score 84.9±4.2, forced vital capacity (FVC) 85±19% pred and FEV1 70±24% pred.

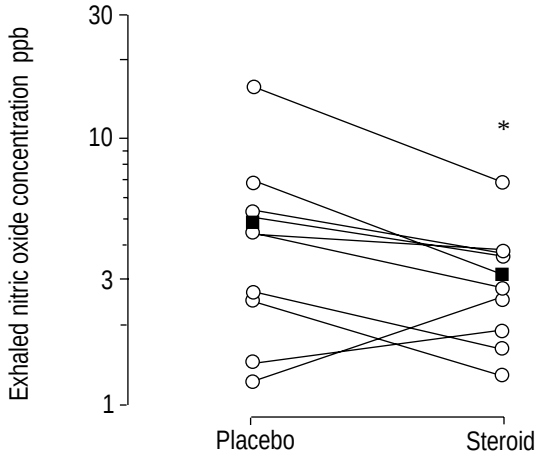

Fig. 1. – Effect of oral glucocorticoid treatment on exhaled nitric oxide concentration in cystic fibrosis patients. The values for each patient during placebo and glucocorticoid treatments are joined by a straight line. ■: mean value during placebo = 4.9; ■: mean value during steroid treatment = 3.1. *: $p < 0.05$ *versus* placebo treatment.

Mean exhaled NO decreased significantly following glucocorticoid treatment (fig. 1), with eight of the 10 patients showing a reduction in exhaled NO. The two patients in which exhaled NO rose had the lowest concentrations while on placebo. Spirometric indices were not altered following prednisolone treatment (table 1). Peripheral blood differential white cell counts showed an increase in mean concentration of neutrophils and a decrease in eosinophils after glucocorticoid treatment (table 1). There was no

Table 1. – Spirometric indices and blood parameters for placebo and glucocorticoid treatments

Parameter	Placebo	Glucocorticoid
FVC L	4.24±1.29	4.22±1.34
FEV_1 L	2.95±1.16	2.94±1.17
$FEF_{25-75\%}\ L \cdot min^{-1}$	145±92	146±90
PEF $L \cdot min^{-1}$	439±114	435±147
WCC $10^9 \cdot L^{-1}$	8.3±1.2	11.2±2.0**
Neutrophil count $10^9 \cdot L^{-1}$	5.8±1.5	9.4±2.0**
Eosinophil count $10^9 \cdot L^{-1}$	0.3±0.2	0.1±0.1**
CRP $mg \cdot L^{-1}$	9.7±8.0	7.8±7.6
Serum $NO_2/NO_3\ \mu mol \cdot L^{-1}$	12.4±9.1	13.0±12.2

Data are presented as mean±SD; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; $FEF_{25-75\%}$: mean expiratory flow over 25–75% FVC; PEF: peak expiratory flow; WCC: peripheral blood total white cell count; CRP: C-reactive protein; NO_2/NO_3: nitrite/nitrate oxide. **: $p < 0.01$ *versus* placebo treatment.

change in the mean serum NO_2/NO_3 or CRP after each treatment period. The responses in the three patients on inhaled glucocorticoids prior to study entry were similar to those of the remainder of the patient group.

Discussion

The low exhaled NO concentrations observed in the present study during placebo ingestion are in agreement with previous reports in CF patients [1–3]. Glucocorticoid treatment reduced the exhaled NO further in these stable patients (fig. 1). The reduction in concentration was in excess of 30% of basal values. Since all measurements were carried out at constant flow, this represents an exactly equal reduction in the total amount of NO exhaled. NO is a highly reactive gas that is rapidly oxidized by reaction with oxygen and superoxide radical at or close to its site of production [17]. Therefore, only a small proportion of the total NO that is produced will reach the airway lumen, in particular in the presence of airway inflammation. Thus, the measured reduction in exhaled NO underestimates the total reduction in NO production in the cells of the airway epithelium.

Glucocorticoid treatment produced the expected effects on peripheral blood cell counts [18], increasing total cell and peripheral neutrophil counts and reducing the eosinophil count (table 1), confirming that the patients complied with the study protocol.

Glucocorticoids have been shown to reduce the expression of iNOS but not the constitutive NOS (cNOS) isoforms *in vitro* [10]. In asthmatic subjects, exhaled NO is increased due to the activity of iNOS and glucocorticoid treatment reduces both iNOS expression and exhaled NO [13, 14]. The observation that a similar dose of oral glucocorticoid reduced exhaled NO in CF subjects, suggests that iNOS activity contributes to the exhaled NO in CF patients. Although iNOS expression in CF airway epithelium is reduced, its expression in the subepithelial tissues is increased [5]. Glucocorticoid suppression of this subepithelial iNOS could account for the decreased NO excretion observed in the present study.

CF is characterized by a massive neutrophil influx into the lung tissue and since these cells express iNOS, they are a potential source of the exhaled NO. There is evidence that glucocorticoids may reduce neutrophil migration into the airways, a further mechanism that could reduce the amount of iNOS present in the CF airways. However, at the dose of oral glucocorticoid used in the present study, this effect is unlikely to be significant [19]. Nonetheless, it is a mechanism that may have contributed to the decrease in exhaled NO.

Given the ability for strains of Pseudomonas to respire anaerobically by means of denitrification [20], it has been suggested that changes in NO_2/NO_3 in CF patients infected with *P. aeruginosa* should not be interpreted without reference to changes in bacterial load [7]. Since NO is a reaction intermediate in this process, and all patients in the study had positive *P. aeruginosa* sputum cultures, bacterial denitrification is another potential source of NO in CF airways. However, a reduction in the number of bacteria would be required to account for the observed fall in exhaled NO. Given the well-known immunosuppresive effects of glucocorticoids, this seems unlikely.

In conclusion, following a short course of oral glucocorticoid treatment, exhaled nitric oxide was reduced when compared to placebo administration in adults with stable cystic fibrosis. The most plausible explanation for this finding is that a proportion of nitric oxide detectable in the breath of stable cystic fibrosis patients, is due to the presence of the glucocorticoid-suppressible isoform of nitric oxide synthase in the patients' airways.

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References

1. Balfour-Lynn IM, Laverty A, Dinwiddie R. Reduced upper airway nitric oxide in cystic fibrosis. *Arch Dis Child* 1996; 75: 319–322.
2. Linnane SJ, Keatings VM, Costello CM, *et al.* Total sputum nitrate plus nitrite is raised during acute pulmonary infection in cystic fibrosis. *Am J Respir Crit Care Med* 1998; 158: 207–212.
3. Lundberg JO, Nordvall SL, Weitzberg E, Kollberg H, Alving K. Exhaled nitric oxide in paediatric asthma and cystic fibrosis. *Arch Dis Child* 1996; 75: 323–326.
4. Kelley TJ, Drumm ML. Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. *J Clin Invest* 1998; 102: 1200–1207.
5. Meng QH, Springall DR, Bishop AE, *et al.* Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *J Pathol* 1998; 184: 323–331.
6. Ho LP, Innes JA, Greening AP. Nitrite levels in breath condensate of patients with cystic fibrosis is elevated in contrast to exhaled nitric oxide. *Thorax* 1998; 53: 680–684.
7. Grasemann H, Ioannidis I, Tomkiewicz RP, de Groot H, Rubin BK, Ratjen F. Nitric oxide metabolites in cystic fibrosis lung disease. *Arch Dis Child* 1998; 78: 49–53.
8. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87: 1620–1624.
9. van der Vliet A, Eiserich JP, Shigenaga MK, Cross CE. Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 1999; 160: 1–9.
10. Radomski MW, Palmer RM, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular

endothelial cells. *Proc Natl Acad Sci USA* 1990; 87: 10043 – 10047.
11. Jatakanon A, Kharitonov S, Lim S, Barnes PJ. Effect of differing doses of inhaled budesonide on markers of airway inflammation in patients with mild asthma. *Thorax* 1999; 54: 108 – 114.
12. Kharitonov SA, Yates DH, Barnes PJ. Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. *Am J Respir Crit Care Med* 1996; 153: 454 – 457.
13. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *Faseb J* 1998; 12: 929 – 937.
14. Yates DH, Kharitonov SA, Robbins RA, Thomas PS, Barnes PJ. Effect of a nitric oxide synthase inhibitor and a glucocorticosteroid on exhaled nitric oxide. *Am J Respir Crit Care Med* 1995; 152: 892 – 896.
15. American Thoracic Society. Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children. *Am J Respir Crit Care Med* 1999; 160: 2104 – 2117.
16. Verdon CP, Burton BA, Prior RL. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. *Anal Biochem* 1995; 224: 502 – 508.
17. Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 1994; 149: 538 – 551.
18. Baxter JD. Glucocorticoid hormone action. *Pharmacol Ther* 1976; 2: 605 – 669.
19. Keatings VM, Jatakanon A, Worsdell YM, Barnes PJ. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* 1997; 155: 542 – 548.
20. Goretski J, Zafiriou OC, Hollocher TC. Steady-state nitric oxide concentrations during denitrification. *J Biol Chem* 1990; 265: 11535 – 11538.