Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma

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Abstract

***Background* – Eosinophils in induced sputum and exhaled nitric oxide (NO) are currently used as non-invasive markers in the assessment of airway inflammation in asthma. As both sputum eosinophils (%) and exhaled NO are raised in asthmatic subjects not receiving inhaled steroids and decreased following corticosteroid therapy, a relationship between them is plausible.**

***Methods* – Exhaled NO was measured by chemiluminescence analyser, sputum induction by 3.5% saline inhalation, and bronchial responsiveness was measured as $PC_{20}FEV_1$ methacholine in 35 stable asthmatic patients using β_2 agonist alone and the correlation between these non-invasive markers of airway inflammation was studied.**

***Results* – There were significant correlations between exhaled NO and PC_{20} ($r = -0.64$), exhaled NO and sputum eosinophils (%) ($r = 0.48$), and also between sputum eosinophils (%) and PC_{20} ($r = -0.40$).**

***Conclusion* – The correlation between exhaled NO and PC_{20} suggests that exhaled NO or the mechanisms leading to its increase may contribute to airway hyperresponsiveness in asthma. Furthermore, the relationship between sputum eosinophils (%), exhaled NO, and PC_{20} highlight the potential use of eosinophils (%) in induced sputum and exhaled NO to monitor the severity of asthma.**

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Keywords: asthma, induced sputum, eosinophils, exhaled nitric oxide, bronchial hyperresponsiveness.

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The clinical management of asthma relies on monitoring lung function and symptoms. However, airway inflammation may be present in asthmatic patients whose lung function is normal and clinically well controlled,[1] suggesting that these measures may not be sensitive enough to reflect the extent of airway inflammation.

Inflammation in the airways may be reflected by the levels of nitric oxide (NO) in exhaled air. Airway inflammation following induced allergen challenge is accompanied by an elevation in exhaled NO levels,[2] and increased levels of exhaled NO are also seen in asthmatic exacerbations.[3] Exhaled NO levels are increased in patients not treated with inhaled corticosteroids[4] and the levels are not modulated by bronchodilator therapy.[5] However, in asthmatic subjects receiving inhaled corticosteroids the levels are reduced.[6] This suggests that exhaled NO may be used as a surrogate marker of airway inflammation.

The number of sputum eosinophils and the amount of eosinophil cationic protein (ECP) in induced sputum is associated with asthma severity. There are correlations between sputum eosinophils and sputum ECP levels with FEV_1.[7–9] Also, sputum obtained from asthmatics under exacerbation contain very high numbers of eosinophils[7 10 11] but they are reduced following corticosteroid treatment.[12 13] This evidence justifies the validity of using sputum eosinophil number or sputum levels of ECP to monitor asthma severity.

Bronchial hyperresponsiveness (BHR), an exaggerated bronchoconstrictor response to inhaled stimuli, is a key feature of asthma and may be used as an indicator of asthma severity. BHR relates closely to the severity of asthma, the frequency of symptoms, and the need for treatment.[14]

The aim of our study was to examine the relationship between exhaled NO and other non-invasive markers of inflammation, including the number of eosinophils, the amount of ECP in induced sputum, and BHR, and also its relationship with % predicted FEV_1. Such a correlation would allow us to evaluate the clinical utility of exhaled NO and its potential as a surrogate marker of airway inflammation in asthma.

Methods

SUBJECTS

Thirty five atopic asthmatic patients who were using inhaled β_2 agonist alone were studied. All of them had stable asthma which was defined as no changes in asthmatic symptoms and asthma medications in the preceding three months. All had a history of wheezing and chest tightness, were previously diagnosed by a physician as having asthma, had BHR to methacholine producing a 20% fall in FEV_1 (PC_{20}) of ≤ 8 mg/ml, and were using inhaled β_2 agonists alone in order to control asthma. Exclusion criteria were cigarette smoking, history of upper respiratory tract infection within the preceding four weeks, and the use of inhaled or oral steroids within the previous three months. The study protocol was approved by the ethics committee of the Royal Brompton Hospital.

STUDY DESIGN

This was a cross sectional study. Patients were not allowed to use inhaled β_2 agonist for at least six hours before coming to the laboratory. On arrival, all were questioned about their asthmatic symptoms and the average requirement of rescue β_2 agonist used daily within the previous three months. Exhaled NO, spirometric values, methacholine challenge test and sputum induction were then measured. Sputum induction was performed 15 minutes after patients recovered from methacholine challenge test. The patients were required to record on diary cards for seven days (1) peak flow rates in the morning and evening (best of three) using a mini-Wright peak flow meter, (2) symptom scores (asthma during the day, asthma during the night, and early morning tightness, scored 0–3 for each item), and (3) the number of puffs of rescue β_2 agonist required daily. Twelve patients performed a second sputum induction one week after the first to evaluate the effect of methacholine on the cell profile and ECP levels of the induced sputum.

NITRIC OXIDE MEASUREMENT

End-exhaled NO was measured by a chemiluminescence analyser (Model LR2000, Logan Research, Rochester, UK) using the method described by Kharitonov *et al*.[15] In brief, subjects exhaled slowly with an exhalation flow of 5–6 l/min from total lung capacity over 30–40 s through a mouthpiece. NO was sampled from a side arm attached to the mouthpiece. The mean value was taken from the point corresponding to the plateau of end-exhaled CO_2 reading (5–6% CO_2), representing the lower respiratory tract sample. Results of the analyses were computed and graphically displayed on a plot of NO and CO_2 concentrations, pressure, and flow against time.

LUNG FUNCTION

FEV_1 and FVC were measured with a dry spirometer (Vitalograph Ltd, Buckingham, UK) and the best value of the three manoeuvres was expressed as percentage of predicted value.

BRONCHIAL RESPONSIVENESS

PC_{20} was measured by inhalation methacholine challenge with doubling concentrations of methacholine (0.06–32 mg/ml) delivered by dosimeter (Mefar, Bovezzo, Italy) with an output of 100 µl. The aerosols were inhaled at tidal breathing with the patient wearing a noseclip. A total of five inhalations of each concentration was administered (inhalation time one second, breath holding time six seconds) and FEV_1 was measured two minutes after the last inhalation until there was a fall in FEV_1 of 20% compared with the control inhalation (0.9% saline solution) or until the maximal concentration was inhaled. The PC_{20} value was calculated by interpolation of the logarithmic dose-response curve. Subjects were given two puffs (200 µg) of salbutamol by a metered dose inhaler following the methacholine challenge test.

SPUTUM INDUCTION AND PROCESSING

After recovery subjects were instructed to wash their mouths thoroughly with water and 3.5% saline at room temperature nebulised via an ultrasonic nebuliser (DeVilbiss Co, Heston, UK) at maximum output was then inhaled. The subjects were instructed to cough deeply after five and three minute intervals thereafter. The total induction time was 15 minutes. Mouth washing between and before each cough was encouraged in order to minimise salivary contamination. The initial sample from the first cough was discarded. Induced sputum was collected into a 50 ml polypropylene tube kept at 4°C and processed within two hours.

Spirometric tests were repeated after the sputum induction. If FEV_1 had dropped below 15% of the post-salbutamol value the subject was required to stay until it had returned to the baseline value.

For sputum processing 2 ml Hank's balanced salt solution (HBSS) containing 1% dithiothreitol (DTT) (Sigma Chemicals, Poole, UK) was added, vortexed, and repeatedly aspirated at room temperature until the sputum was homogenised. The sputum volume was then recorded. Samples were then further diluted with HBSS up to 10 ml, vortexed briefly and centrifuged at 400*g* for 10 minutes at 4°C.

The supernatant fluid was kept at −70°C for subsequent assay for ECP. The cell pellet was resuspended. Total cell count was performed on a haemocytometer using Kimura stain. An adequate specimen was defined as one having a number of squamous epithelial cells less than 50% of the inflammatory cells. Slides were then prepared using a cytospin (Shandon, Runcorn, UK) and stained with May-Grunwald-Giemsa for differential cell counts which were performed by an observer blind to the clinical characteristics of the subjects. At least two slides were used for counting and at least 300 inflammatory cells were counted in each slide.

ECP ANALYSIS

The concentration of ECP was measured by radioimmunoassay (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Reproducibility of the assay was good with a coefficient of variation of <8%. The detection limit of the assay was <2 mg/l.

STATISTICAL ANALYSIS

Log transformed values of PC_{20} were used for analysis. The mean values of peak flow variability (amplitude % max) and total symptom scores were averaged from the seven day records. Correlations were determined using Spearman rank correlation. A paired sample *t* test was used to analyse the effect of methacholine on sputum inflammatory cell profiles and ECP concentrations, a p value of <0.05 being considered significant.

Table 1 Patient characteristics and exhaled nitric oxide

Patient no.	*Sex*	*Age*	*FEV_1 (% pred)*	*PC_{20} (mg/ml)*	*NO (ppb)*	*Peak flow variability (%)*	*Symptom scores*
1	M	28	90.5	0.75	15.0	5.4	0
2	F	24	111.1	0.32	18.5	5.4	1.6
3	F	31	86.7	2.69	10.5	8.7	0.4
4	F	27	98.4	1.41	9.0	4.9	0
5	F	28	90.5	0.66	14.0	2.9	1.3
6	M	25	115.9	0.06	32.5	5.3	0.0
7	F	29	91.1	0.06	32.5	5.6	1.9
8	F	36	97.1	0.50	15.0	2.6	0
9	M	32	98.6	0.09	33.5	3.3	0
10	F	23	109.0	0.57	28.3	3.1	0
11	F	25	96.0	1.16	16.0	3.6	0
12	M	27	92.7	0.64	24.0	7.4	1.4
13	M	30	88.7	2.42	14.0	3.3	0.3
14	M	28	87.0	2.21	19.0	6.8	1.0
15	M	25	95.3	0.06	40.0	7.5	2.6
16	M	28	90.8	0.41	37.0	3.0	0.4
17	M	22	99.6	0.50	26.0	4.2	0.9
18	F	19	70	0.18	24.0	4.1	1.6
19	M	23	95	0.18	12.5	3.6	0.2
20	M	26	90	0.21	17	3.2	0.4
21	M	20	66	0.15	11	13.9	1.7
22	M	42	108	1.28	16	8.4	2.0
23	M	26	56	0.06	25	28.1	3.8
24	F	23	51	1.01	17	14.5	3.1
25	M	22	84	0.51	10	7.6	0.2
26	M	24	88.3	0.65	28	2.4	1.7
27	F	21	72.8	0.20	41	1.4	3.0
28	F	24	97.4	0.33	18.5	11.2	0.4
29	M	22	58.4	0.12	26	20.9	1.9
30	F	25	88.9	3.37	7	9.4	2.6
31	M	25	76.1	4.32	8	1.5	0.3
32	M	19	91.9	4.82	8	1.6	0.9
33	F	24	79.6	1.62	13	7.5	3.0
34	M	21	100.9	0.29	14.5	6.7	1.0
35	M	24	101	0.80	13.4	7.4	1.1
Mean (SE)		25.6 (0.8)	88.9 (2.6)	0.52 (1.23)*	19.9 (1.6)	6.8 (0.9)	1.1 (0.2)

FEV_1 = forced expiratory volume in one second; PC_{20} = concentration of methacholine required to produce a 20% drop in FEV_1; ppb = part per billion; *geometric mean (SE).

Table 2 Sputum characteristics and sputum ECP levels

Patient no.	*Volume (ml)*	*TCC/ml ($\times 10^6$)*	*Mac (%)*	*Neu (%)*	*Eo (%)*	*Lym (%)*	*ECP (ng/ml)*
1	4.9	1.51	30.0	70.0	0	0	12.7
2	3.35	0.97	70.5	29.0	0.5	0	25.3
3	2.5	0.73	73.2	25.3	0.5	1.0	32.8
4	2.9	0.43	61.2	34.5	3.3	1.0	42.7
5	2.4	0.52	75.4	17.3	7.3	0	156.2
6	2.5	0.56	76.7	14.8	8.2	0.3	38
7	6.75	0.25	68.0	16.0	16.0	0	27.4
8	2.15	0.98	80.2	18.3	0.8	0.7	<2
9	5.1	0.81	63.4	33.3	3.3	0	25.1
10	3.4	0.37	42.8	55.5	1.7	0	12.1
11	2.35	0.51	57.1	42.4	0.4	0	24.0
12	3.25	1.37	80.8	18.2	1.0	0	191.3
13	2.7	0.22	37.8	55.7	6.5	0	60.7
14	3.25	0.42	47.7	42.3	8.0	2.0	52
15	3.75	0.53	67.5	14.7	16.2	1.7	338.6
16	5.7	0.63	80.3	5.0	11.7	3.0	37
17	3.0	1.81	92.0	4.2	3.5	0.3	307
18	2.65	2.37	40.8	41.0	15.8	2.5	135.8
19	3.2	0.69	27.7	71.6	0.2	0.5	40.9
20	2.05	2.16	47.4	52.4	0.2	0.0	128.2
21	1.0	4.05	17.5	80.3	2.0	0.2	20.2
22	9.3	2.43	86.7	12.3	1.0	0.0	60.7
23	2.8	1.58	42.0	48.3	3.0	6.7	22.0
24	5.8	5.34	88.3	5.7	5.3	0.7	46.9
25	4.3	1.78	83.3	13.0	3.3	0.4	12.4
26	5.6	1.2	65.3	24	7	3.7	34
27	1.1	0.26	36	38	15	11	23.5
28	7.6	3.8	42.7	40.3	16.7	0.3	41.4
29	4.1	1.3	79.0	8.3	8.7	4.0	38.2
30	4.5	2.0	75.7	21	1	2.3	13.8
31	2.2	4.9	48.0	44.7	4.6	2.7	62.1
32	4.4	1.3	71.7	25.7	1.6	1.0	<2
33	1.0	2.6	73	18.7	5.6	2.7	30.3
34	1.5	2.8	76.3	17.1	6.6	1.0	13.1
35	0.6	1.6	57.7	32.3	6.6	3.4	114
Median	3.2	1.30	67.5	25.7	3.5	0.7	38.0
IQR	2.3–4.4	0.53–2.16	42.8–76.7	16.0–42.4	1.0–8.0	0–2.5	23.7–61.4

TCC = total cell count; Mac = macrophages; Neu = neutrophils; Eo = eosinophils; Lym = lymphocytes; ECP = eosinophil cationic protein; IQR = interquartile range.

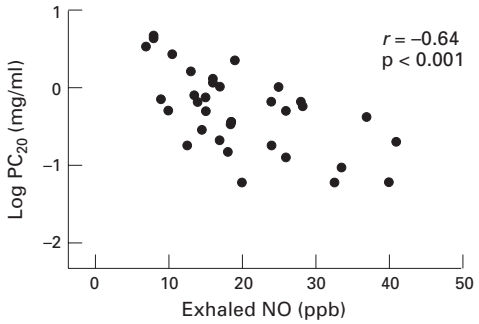

Figure 1 Correlation between exhaled nitric oxide (NO) and the concentration of methacholine required to produce a 20% drop in FEV_1 (PC_{20}).

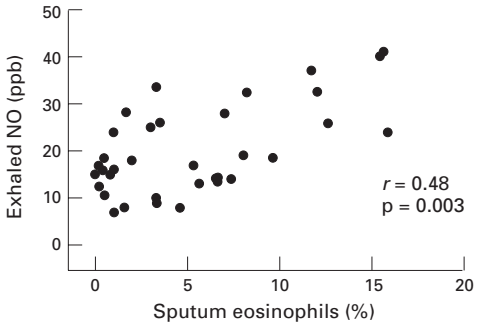

Figure 2 Correlation between exhaled NO and sputum eosinophils (%).

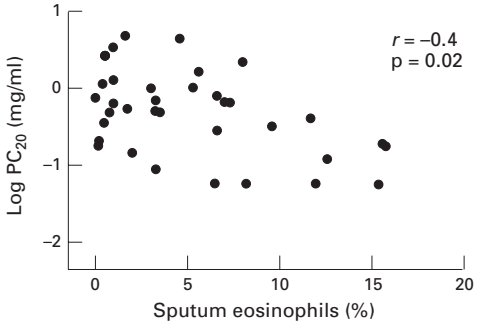

Figure 3 Correlation between sputum eosinophils (%) and PC_{20}.

Results

Most of our patients had mild asthma based on conventional terms, as demonstrated by low symptom scores, low variability of peak flow, and normal FEV_1. Only a few (patients 23, 24, and 29) had moderately severe asthma (table 1). No patients developed significant bronchospasm after sputum induction. The exhaled NO level was increased compared with normal subjects (table 1). Sputum eosinophils and sputum ECP levels were raised, ranging from 0 to 16.7% and 0 to 338 ng/ml, respectively (table 2).

Methacholine had no effect on sputum inflammatory cell profiles and ECP levels, with no differences between specimens collected immediately and one week after the methacholine challenge test (table 3). The reproducibility of the sputum differential cell count in asthmatic patients performed in our laboratory showed an intraclass correlation coefficient of 0.75 for eosinophils, 0.78 for neutrophils, 0.76 for macrophages, and 0.56 for lymphocytes.

Table 3 Mean (SE) sputum inflammatory cell profiles and ECP immediately after the methacholine challenge test and one week later in 12 asthmatic patients

	Immediately after methacholine	*One week after methacholine*
Sputum volume (ml)	3.6 (0.6)	3.7 (0.7)
TCC × 10^6/ml	3.09	2.49
Macrophages (%)	61.6 (6.1)	60.7 (6.9)
Neutrophils (%)	28.5 (5.5)	26.9 (5.2)
Eosinophils (%)	6.8 (1.2)	6.3 (1.4)
Lymphocytes (%)	2.9 (6.1)	3.8 (2.6)
ECP (ng/ml)	32.1 (8.8)	34.4 (8.2)

TCC = total cell count; ECP = eosinophil cationic protein.

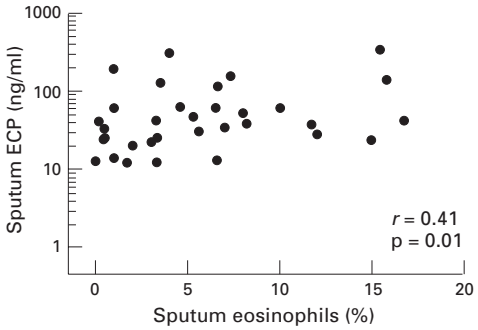

Figure 4 Correlation between sputum eosinophils (%) and sputum ECP levels.

CORRELATIONS

There were significant correlations between exhaled NO and PC_{20} ($r = -0.64$, $p<0.001$, fig 1), exhaled NO and sputum eosinophils (%) ($r=0.48$, $p=0.003$, fig 2), sputum eosinophils (%) and PC_{20} ($r = -0.40$, $p=0.02$, fig 3), and sputum eosinophils and ECP levels ($r=0.41$, $p=0.01$, fig 4). No significant relationships were found between exhaled NO, sputum eosinophils (%), sputum ECP, or PC_{20} and FEV_1, peak flow variability or symptom scores.

Discussion

A significant correlation was found between exhaled NO, PC_{20}, and sputum levels of eosinophils. These relationships suggest that NO may be useful as a marker of airway inflammation in asthma, as NO itself – or the mechanisms resulting in its increase – may contribute to airway inflammation in asthma.

There is evidence to suggest that NO itself may contribute to airway hyperresponsiveness or inflammation. At high concentrations NO may increase airway oedema since it is a potent vasodilator and increases plasma exudation from airway vessels.[16] Furthermore, an animal model of asthmatic inflammation indicates that NO may increase airway inflammation by enhancing the recruitment of eosinophils into the airways.[17] Internal formation of NO may result in the formation of peroxynitrite through interaction with superoxide anions.[18] Peroxynitrite induces airway hyperresponsiveness in guinea pigs.[19] However, in two previous studies there was no apparent correlation between exhaled NO and PC_{20}.[20 21] Failure to demonstrate such a relationship could have been due to either a small sample size or narrow ranges of the levels of exhaled NO and PC_{20} measured, or both.

The correlation between exhaled NO and sputum eosinophils, even though not strong, suggests a causal link. A possible explanation could be that NO derived from airway epithelial cells and inflammatory cells is increased due to induction of NO synthase (NOS) by the exposure to pro-inflammatory cytokines such as TNF-α and IL-1β secreted by macrophages. The inducible form of NOS has a much greater capacity to produce NO than the constitutive form. Such a large amount of NO has been reported to result in suppression of Th1 cells and a concomitant reduction in the level of IFNγ, leading to a proliferation of Th2 cells. Th2 cells then produce several cytokines including IL-5 which is important in the recruitment of eosinophils into the airways.[22].

There is evidence to suggest that PC_{20} relates closely to the severity of asthma and the need for treatment.[14] The wide range of PC_{20} in mild asthma may limit its use as a sensitive marker of airway inflammation. Nevertheless, we have demonstrated a relationship between sputum eosinophils with PC_{20} over a wide range. This wide range may indicate that a varying degree of airway inflammation can be found in patients with mild asthma, despite the fact that the clinical severity as judged by spirometric tests, peak flow variability, and symptoms is similar.

Monitoring of eosinophils in sputum may also be useful as their numbers reflect the extent of airway inflammation.[7 8] However, eosinophil numbers in sputum can also vary widely in patients with the same clinical severity of asthma. This suggests that estimation of the extent of airway inflammation should not be based on either PC_{20} or sputum eosinophil numbers alone. Furthermore, their relationship with exhaled NO indicates that these markers may complement each other in determining asthma severity in patients using inhaled β_2 agonists alone.

The great advantage of exhaled NO is that the measurement is completely non-invasive, it can be performed repeatedly, and it can be used in children and patients with severe airflow obstruction where more invasive techniques are not possible.[23] The measurement is not specific, however, as exhaled NO is also increased in inflammation due to bronchiectasis and respiratory tract infections. This means that serial measurements in individual patients could be more useful than single measurements. Furthermore, exhaled NO does not appear to be a good marker of airway inflammation in patients with steroid-dependent asthma (Jatakanon *et al*, unpublished data).

A relationship between eosinophils (%) or ECP and percentage predicted FEV_1 or PC_{20} has been found previously.[7–9] A relationship between sputum eosinophils and PC_{20} has also been shown in our study but we could not detect a correlation between the number of eosinophils or the amount of ECP in induced sputum and FEV_1. This may in part be due to the narrow range of FEV_1 in our patients, and may also explain the weak relationship between sputum eosinophils and ECP levels as most of our patients had mild asthma and therefore had low levels of ECP in induced sputum.

However, contamination of the whole sputum specimen with saliva may also lower ECP levels.

We could not identify any significant relationships between peak flow variability or symptom scores and either PC_{20} or sputum eosinophils. This may indicate a lack of sensitivity of these measurements as a reflection of the minor degree of airway inflammation found in mild asthma.

We conclude that exhaled NO monitoring in asthma may be useful as it complements sputum eosinophil numbers for determining the extent of airway inflammation in asthma. However, long term studies with serial measurements of exhaled NO levels, sputum eosinophils, bronchial responsiveness, and their relationship with symptoms and lung function, either following anti-inflammatory treatment or in patients with unstable asthma, are required to determine their clinical value.

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