

Local nitric oxide levels reflect the degree of allergic airway inflammation after segmental allergen challenge in asthmatics[☆]

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Abstract

Nitric oxide (NO) levels are increased in the exhaled air of asthmatics. As NO levels correlate with allergic airway inflammation, NO measurement has been suggested for disease monitoring. In patients with asthma, we previously demonstrated that intrabronchial treatment with a natural porcine surfactant enhanced airway inflammation after segmental allergen provocation. We studied whether local levels of NO reflect the degree of allergic airway inflammation following segmental allergen challenge with or without surfactant pretreatment. Segmental NO, as well as nitrite and nitrate in bronchoalveolar lavage (BAL) fluid, was measured before and after segmental challenge with either saline, saline plus allergen, or surfactant plus allergen in 16 patients with asthma and five healthy subjects. The data were compared with inflammatory BAL cells. Segmental NO levels were increased after instillation of saline ($p < 0.05$), or surfactant plus allergen in asthmatics ($p < 0.05$), and values were higher after surfactant plus allergen compared to saline challenge. Nitrate BAL levels were not altered after saline challenge but increased after allergen challenge ($p < 0.05$) and further raised by surfactant ($p < 0.05$), whereas nitrite levels were not altered by any treatment. Segmental NO and nitrate levels correlated with the degree of eosinophilic airway inflammation, and nitrate levels also correlated with neutrophil and lymphocyte numbers in BAL. In healthy subjects, NO, nitrite, and nitrate were unaffected. Thus, segmental NO and nitrate levels reflect the degree of allergic airway inflammation in patients with asthma. Measurement of both markers can be useful in studies using segmental allergen provocation, to assess local effects of potential immunomodulators.

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Asthma is characterized by chronic airway inflammation and associated with excess production of nitric oxide (NO) which is known to be a key mediator of inflammation involved in vasodilation, bronchodilation, neurotransmission, and cell-mediated immunity [1,2].

If produced in excess, NO can exert cytotoxic effects, especially in combination with other reactive molecular species [3]. NO can be easily detected in the exhaled air of human subjects. As its concentration depends on measurement conditions [4], guidelines have been established to standardize measurements to generate reproducible and comparable data that can be used for clinical decision making [5].

The concentration of exhaled NO is increased in patients with untreated asthma [6–9], whereby elevated

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levels are associated with both chronic inflammation [10] and acute responses to allergen inhalation [11,12]. In line with this, exhaled NO correlates with eosinophil numbers in BAL fluid [13] or sputum [14], as well as with eosinophilic infiltration in bronchial biopsies [15]. Glucocorticoid treatment, which attenuates airway inflammation, also reduces NO levels [16,17]. Based on this, it has been suggested that exhaled NO is an effective, non-invasive measure for monitoring asthma [18]. Besides total NO in exhaled breath, local NO levels which can be determined during bronchoscopy have also been found elevated after segmental allergen provocation [12,19].

Previous studies have shown that pulmonary surfactant plays a role in asthma and allergic inflammation [20]. Surfactant can attenuate allergen-induced lymphocyte proliferation in children with asthma [21], and surfactant proteins SP-A and SP-D can downregulate allergic inflammation in mice [22,23]. The administration of exogenous surfactant for asthma therapy is under current investigation [24]. In a recent study, we tested whether treatment of asthmatics with a natural porcine surfactant modulated the response to segmental allergen challenge [25]. Surprisingly, we found that this exogenous surfactant preparation augmented eosinophilic inflammation.

To better characterize these effects, we additionally investigated whether local NO levels were raised by surfactant in combination with allergen challenge. Furthermore, we analyzed whether the resulting NO levels would be related to those of nitrite and nitrate or differential cell numbers in bronchoalveolar lavage (BAL) fluid, or to exhaled NO as a marker that is easily measured but integrating over the whole lung. Besides providing information on the immunomodulatory effect of surfactant, such data could help to assess whether measurement of segmental versus exhaled NO is sensitive and applicable for detecting local inflammatory effects, and whether local airborne NO versus soluble surrogate markers would yield different results.

Methods

Study design

Sixteen mild asthmatics and five healthy volunteers underwent two bronchoscopies 24 h apart. On day 1, baseline values were assessed and challenges performed. After bronchoalveolar lavage, saline was given segmentally as a control challenge (sham). Ten asthmatic and all healthy subjects were then treated in two other segments with either Curosurf (Nycomed, Munich, Germany) or saline immediately followed by local allergen challenge in the same segments. To assess potential direct effects of Curosurf, the other six asthmatic subjects received

Curosurf alone, without subsequent allergen challenge, instead of saline plus allergen in one of the two segments. On day 2, all subjects were rebronchoscoped and underwent three lavage procedures in the three segments chosen on day 1. Exhaled NO was measured before and 4 h after bronchoscopy on both day 1 and 2. Measurement of exhaled NO and segmental NO levels was performed in 14 asthmatics and five healthy controls. Nitrate and nitrite levels were assessed in all 16 asthmatics and five healthy controls.

Study subjects

Subjects with asthma had a positive skin prick test for grass pollen or house dust mite. They had a history of episodic wheeze with reversible airflow obstruction, and asthma had previously been diagnosed by an independent physician. The median duration of the disease since diagnosis was 12.5 (8.5–17.0) years. According to the asthma classification defined in the GINA Workshop Report 2002 (www.ginasthma.com), patients were classified as mild asthmatics. Six asthma patients received intermittent steroid treatment but none of them was treated with corticosteroids, sodium cromoglycate, theophylline or leukotriene modifiers within 4 weeks before bronchoscopy. The allergen extract used for segmental challenge was either mixed grass pollen or *Dermaphagoides pteronyssinus* (ALK-Scherax, Hamburg, Germany), depending on which one produced the largest wheal in a skin prick test. Allergen concentration was also derived from this test. The healthy volunteers had no history of allergic or other diseases, and allergens used for provocation were chosen randomly. All subjects were non-smokers, with detailed characteristics as given previously [25]. Median values (ranges) for asthmatic and healthy subjects were as follows: age 24.5 (22.5–28.0) and 22 (19.0–24.5) years; FEV₁ 105 (99–110) and 115 (114–119) %predicted; PC₂₀FEV₁ 2.59 (0.75 to >8) and >8 mg/ml methacholine; and IgE 221 (94.5–375) and 14 (8.5–20.5) kU/L, respectively. All subjects gave their written informed consent after the study had been approved by the Ethical Committee of the Hannover Medical School.

Bronchoscopy with segmental allergen challenge and surfactant treatment

Bronchoscopy was performed as previously described [25]. All subjects received nebulized salbutamol (1.25 mg), atropine (0.5 mg s.c.), and midazolam (0.05–0.1 mg/kg i.v.). Lidocaine (maximum: 6 mg/kg) was used to locally anesthetize the upper and lower airways. The bronchoscope (BF 160 P, Olympus Optical, Hamburg, Germany) was advanced into the anterior bronchus of the left lower lobe, where baseline BAL was done with 6 × 20 ml sterile saline. Lavage fluid from the first 20 ml

was discarded. The instrument was passed into the anterior lingular bronchus and 10 ml saline solution was instilled as sham treatment. For all instillations, a Teflon catheter inserted through the working channel of the bronchoscope was used. The bronchoscope was then passed into the medial segment of the middle lobe and 120 mg Curosurf in 10 ml of saline was instilled followed by allergen challenge. Next, the bronchoscope was passed into the anterior segment of the right upper lobe for control treatment with 10 ml saline, with subsequent allergen challenge. In six asthmatic subjects, Curosurf alone (in 10 ml of saline) not followed by allergen was instilled into this segment.

After 24 h all subjects were rebronchoscoped with the same premedication as on day 1. The anterior lingular bronchus (sham), the right anterior upper lobe bronchus (Curosurf alone or saline plus allergen), and the medial middle lobe bronchus (Curosurf plus allergen) were lavaged. BAL fluid samples were processed as described previously [26]; cells were filtered through a 100 μm filter and centrifuged, while the supernatant was aliquoted and stored at -80°C until analysis. The total number of nucleated cells was assessed using a Neubauer hemocytometer. Differential cell counts were obtained on cytospin slides from 300 cells.

Measurement of nitric oxide

Exhaled NO

Exhaled NO was measured online during single exhalations at 50 ml/s according to a standard protocol [27], using a calibrated chemiluminescence analyzer (NOA 280, Sievers, Boulder, CO, USA). The sampling rate was chosen to be 20 Hz.

Segmental NO

In three lung segments NO was measured during bronchoscopy prior to lavage and instillation. The exception was that baseline values on day 1 were obtained in the anterior bronchus of the left lower lobe prior to lavage; these values were used for comparison with the values obtained after sham treatment in the anterior segment of the lingula on day 2. For segmental measurement, a Teflon catheter was introduced through the working channel of the bronchoscope and connected to the NOA 280, working again at a sampling rate of 20 Hz. The catheter tip was placed in the center of the airway lumen under fiber-optic surveillance. Individuals were instructed to hold their breath for 30 s during measurements. During all procedures they breathed room air. NO levels are given as parts per billion (ppb).

Determination of NO_2^- and NO_3^- in BAL fluid

Nitrite and nitrate were detected using the NOA 280 according to the manufacturer's instructions. Briefly, 5 μl

of BAL fluid was injected with a Hamilton syringe into a purge vessel attached to the NOA 280. Nitrite and nitrate were converted to NO using a saturated solution of VCl_3 in 0.8 M HCl. Nitrite alone was converted to NO using a 1% w/v solution of KI in acetic acid. Zero values were measured using N_2 gas. Final concentrations given as $\mu\text{mol/L}$ were calculated using appropriate nitrite and nitrate standards, whereby nitrate levels were derived by subtracting nitrite levels from the combined nitrite/nitrate level.

Statistical analysis

Unless otherwise stated, the data in the text and figures were expressed as median values and quartiles or individual results. The Mann–Whitney U test was used to perform comparisons between the groups of asthmatic and control subjects. To compare data within groups, the Wilcoxon signed-ranks test was used. A Bonferroni correction for multiple comparisons was not applied, instead p values are given explicitly wherever possible. Correlations were assessed by computing Spearman rank correlation coefficients. p values less than 0.05 were considered statistically significant. Statistics were calculated using GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA, USA).

Results

Increased exhaled NO levels after allergen challenge in asthmatics

On day 1, exhaled NO values in asthmatics ($n=14$) at 4 h post-bronchoscopy were slightly lower than values before bronchoscopy ($p=0.011$, Fig. 1A). On day 2 (24 h after allergen challenge), there was no difference between NO levels before and after bronchoscopy ($p=0.119$). However, higher levels of exhaled NO than at day 1 were observed before bronchoscopy ($p=0.011$) and after bronchoscopy ($p=0.001$, Fig. 1A). No significant changes were observed in healthy subjects ($n=5$, Fig. 1B).

Exhaled NO values before bronchoscopy on day 1 and 2, as well as on day 2 after bronchoscopy were higher in asthmatic compared to healthy subjects ($p=0.023$, 0.014, and 0.037, respectively); there was no significant difference between groups on day 1 after bronchoscopy ($p=0.094$).

Increased segmental NO levels after saline challenge and after surfactant treatment plus allergen challenge in asthmatics

Segmental NO levels before challenge on day 1 were compared with segmental NO levels on day 2, 24 h after

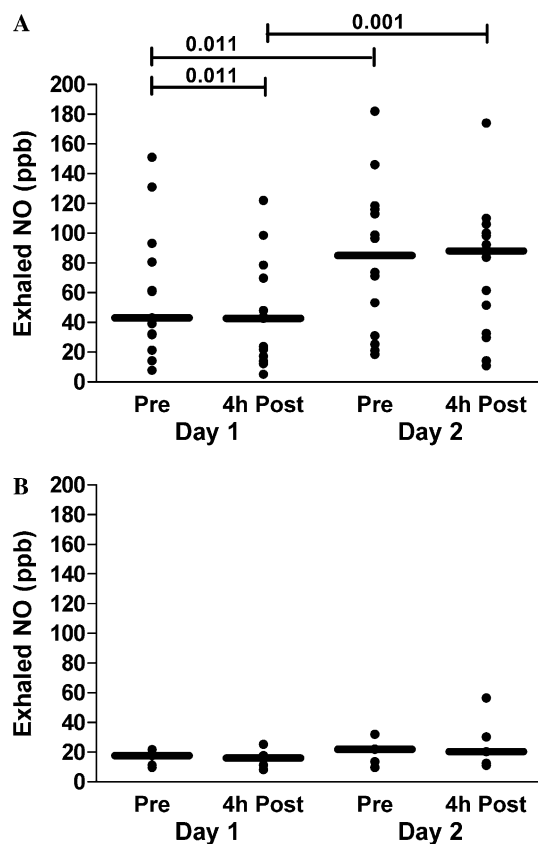


Fig. 1. Exhaled NO in response to allergen. Levels of exhaled NO as measured in asthmatic (A) and healthy subjects (B) before (Pre) and 4 h after (4h Post) bronchoscopy. On day 1, instillations of saline, saline plus allergen, and surfactant plus allergen were done in three segments, and on day 2, bronchoalveolar lavages were performed in the same segments.

allergen challenge. Subjects with asthma demonstrated increased levels of segmental NO at 24 h after challenge with saline ($n=14$, $p=0.011$), and after challenge with surfactant plus allergen ($n=14$, $p=0.011$) while no significant increase was observed in segments challenged with saline plus allergen ($n=8$, $p=0.15$, Fig. 2A). Instillation of surfactant plus allergen led to higher NO levels compared to saline challenge ($p=0.013$), while there was no significant difference against saline plus allergen ($p=0.15$). Surfactant treatment without allergen challenge in six patients did not raise NO levels compared to saline challenge (data not shown). The healthy subjects ($n=5$) showed no significant differences before versus after instillation of saline, saline plus allergen, and surfactant plus allergen, or differences between treatments (Fig. 2B).

Increased nitrate levels after allergen challenge in asthmatics

Nitrite levels were not significantly altered by any of the treatments, neither in asthmatics ($n=16$) nor in healthy subjects ($n=5$, see Table 1).

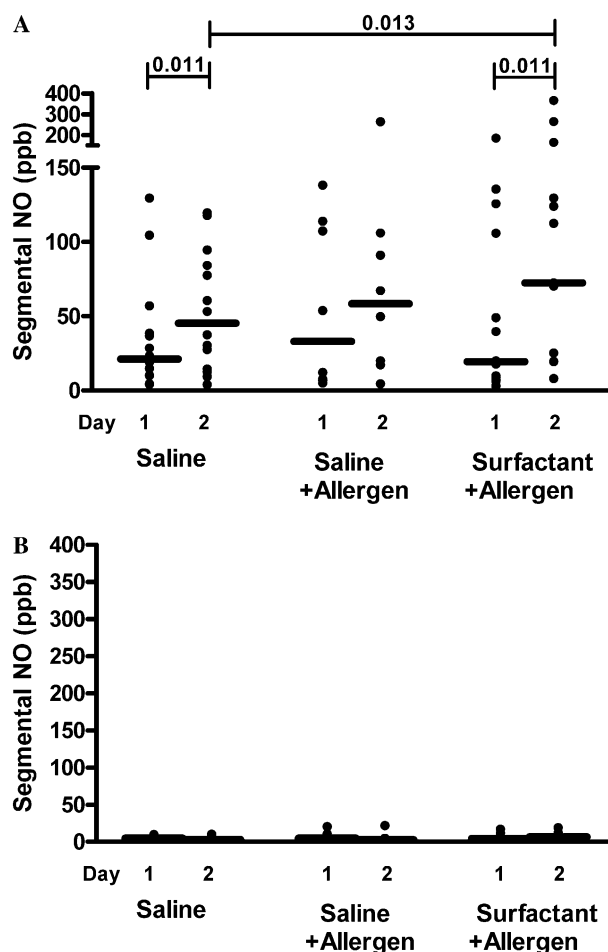


Fig. 2. Segmental NO levels after allergen challenge with and without surfactant pretreatment in asthmatic (A) and healthy subjects (B). Baseline levels of NO were measured in the anterior bronchus of the left lower lobe on day 1. Saline instillation was performed in the anterior segment of the lingula where the NO response was determined on day 2. In addition, NO was measured before (day 1) and 24 h after (day 2) saline plus allergen instillation in the anterior segment of the right upper lobe, as well as before and 24 h after surfactant plus allergen instillation in the medial segment of the middle lobe.

Table 1
Nitrite levels in asthmatic and healthy subjects^a

	Asthma ($\mu\text{mol/L}$)	Healthy ($\mu\text{mol/L}$)
Baseline	0.47 (0.26–0.75)	0.19 (0.02–0.73)
Saline	0.34 (0.26–0.59)	0.53 (0.05–0.71)
Saline plus allergen	0.53 (0.26–1.13)	0.26 (0.11–0.86)
Surfactant plus allergen	0.43 (0.29–0.84)	0.22 (0.11–0.79)

^a Values given are medians. Quartiles are presented in parentheses.

Nitrate levels in asthmatics were increased after allergen challenge ($n=10$) compared to baseline values ($n=16$) on day 1 ($p=0.049$) or compared to saline challenge ($n=16$, $p=0.014$, Fig. 3A). Treatment with surfactant prior to allergen challenge ($n=16$) led to increased nitrate concentrations compared to baseline ($p=0.018$) and compared to saline challenge ($p=0.001$). Nitrate

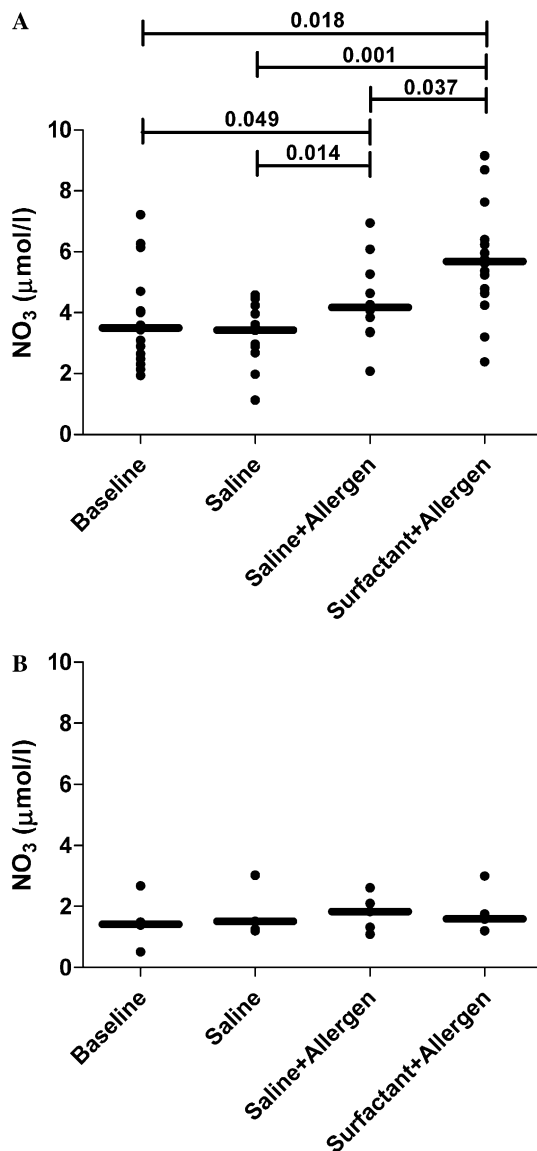


Fig. 3. Nitrate levels in BAL fluid at baseline (day 1) and after instillation of saline, saline plus allergen, and surfactant plus allergen (day 2) in asthmatic (A) and healthy subjects (B).

levels were further raised after surfactant plus allergen compared to saline plus allergen challenge ($p=0.037$, Fig. 3A).

The healthy subjects ($n=5$) did not show significant effects regarding nitrate levels (Fig. 3B). Compared to these subjects, asthmatics showed higher nitrate levels at baseline, after saline plus allergen, and surfactant plus allergen ($p=0.004$, $p=0.003$, and $p=0.002$, respectively).

Segmental NO and nitrate levels correlated with the degree of inflammatory cell influx

As previously shown [25] and summarized in Table 2, eosinophils were virtually absent in healthy subjects and at baseline in subjects with asthma. After allergen challenge plus saline treatment, the number of BAL

eosinophils was significantly raised compared to baseline ($p=0.002$) or saline challenge ($p=0.002$). Surfactant treatment prior to allergen challenge further increased eosinophil numbers ($p=0.004$ compared to allergen challenge) (Table 2).

On day 2, BAL eosinophil numbers correlated with segmental NO levels ($r=0.351$, $p=0.039$, Fig. 4A) and even stronger with BAL nitrate levels ($r=0.585$, $p<0.0001$, Fig. 4B). In addition, a correlation between BAL nitrate levels and BAL lymphocyte numbers ($r=0.575$, $p<0.0001$, Fig. 4C) and BAL neutrophil numbers ($r=0.565$, $p<0.0001$, Fig. 4D) was observed. No correlation was observed between segmental NO levels and neutrophil numbers ($r=0.004$, $p=0.98$), lymphocyte numbers ($r=0.198$, $p=0.25$), or macrophage numbers ($r=-0.157$, $p=0.37$). There was no significant correlation between macrophage numbers and BAL nitrate levels ($r=0.297$, $p=0.057$).

Exhaled and segmental NO levels correlated before and after allergen challenge in asthmatics

In asthmatics, exhaled NO levels measured on day 1 before bronchoscopy were closely related to the median NO levels of the three lung segments prior to instillation ($r=0.962$, $p<0.0001$). Twenty-four hours after allergen challenge, exhaled NO levels measured before bronchoscopy also correlated with median NO levels from three challenged segments ($r=0.833$, $p=0.0002$).

Discussion

The present study demonstrates the relationship between segmental NO levels and total exhaled NO as well as markers of local airway inflammation in allergic asthma, either under baseline conditions or after local enhancement of inflammation by different stimuli. The augmentation of allergen-induced inflammation by natural porcine surfactant, as shown for BAL eosinophils [25], could also be detected when analyzing segmental NO levels and the concentration of nitrate in BAL fluid.

Increased NO levels were observed 24 h after local challenges in asthmatics, and this increase was reflected in the concentration of total exhaled NO. Local NO values after surfactant plus allergen challenge were even higher compared to saline challenge although the saline challenge itself induced an increase of segmental NO levels. NO levels after allergen challenge with saline treatment showed only a tendency towards an increase; the lack of significance was probably due to the reduced sample size, as we decided to treat the respective segment with surfactant alone in six of the 16 asthmatics. Data from these six subjects demonstrated that there were no effects of surfactant itself.

Table 2
Total and differential BAL cell count

Subjects	BAL	n	Total cells ($\times 10^6$)	Eosinophils ($\times 10^6$)	(%)	Neutrophils ($\times 10^6$)	(%)	Lymphocytes ($\times 10^6$)	(%)	Macrophages ($\times 10^6$)	(%)
Healthy	Baseline	5	6.0 (4.0–9.8)	0.0 (0.0–0.1)	0.0 (0.0–1.0)	0.2 (0.1–0.2)	3.0 (2.5–3.0)	0.4 (0.3–0.9)	10.0 (6.5–10.0)	5.5 (3.5–8.7)	87.0 (86.5–90.0)
	Saline	5	5.5 (4.1–14.3)	0.1 (0.0–0.1)	1.0 (1.0–1.0)	1.0 (0.2–4.0)	16.0 (5.0–25.0)	0.5 (0.4–1.2)	9.0 (7.5–10.5)	4.0 (3.4–9.0)	76.0 (65.5–84.0)
	Saline + allergen	5	8.5 (5.8–29.0)	0.1 (0.1–0.3)	1.0 (0.0–1.0)	2.6 (0.6–15.0)	25.0 (10.0–48.5)	0.9 (0.5–1.6)	8.0 (6.0–10.5)	4.9 (4.6–12.3)	66.0 (43.0–80.0)
Asthma	Surfactant + allergen	5	6.6 (5.0–87.0)	0.1 (0.0–1.1)	1.0 (0.5–1.5)	2.2 (0.6–61.0)	33.0 (12.5–67.5)	0.6 (0.4–4.0)	7.0 (4.5–9.5)	4.4 (3.6–20.3)	56.0 (25.0–79.0)
	Baseline	16	4.5 (3.5–6.6)	0.0 (0.0–0.1)	1.0 (0.8–1.0)	0.1 (0.1–0.2)	2.5 (1.5–4.0)	0.6 (0.3–0.9)	11.0 (8.5–16.5)	3.7 (2.9–5.3)	83.0 (78.5–88.5)
	Saline	16	7.1* (5.7–10.7)	0.1 (0.0–0.2)	1.0 (0.5–1.0)	1.0** (0.3–4.0)	12.5** (5.5–45.5)	0.7 (0.5–1.3)	9.5* (8.0–12.0)	4.7 (2.9–6.3)	76.0** (40.0–82.5)
	Saline + allergen	10	28.8*** (21.5–62.0)	19.5*** (5.6–43.9)	64.0*** (29.5–76.0)	4.6*** (1.0–9.8)	12.5** (4.5–28.0)	2.0** (1.1–5.4)	7.5* (4.5–12.5)	6.1 (2.9–9.4)	17.5*** (7.5–31.0)
	Surfactant + allergen	16	68.0*** (44.3–227.5)	39.8*** (22.4–148.8)	61.0*** (49.0–71.0)	14.0*** (6.4–32.6)	15.0** (8.5–27.5)	7.5*** (3.6–15.0)	9.0* (5.0–10.5)	8.2** (5.3–17.0)	11.0*** (8.0–16.5)

Fluid recovery, cell counts, and percentage of cells in bronchoalveolar lavage fluid from healthy control and asthmatic subjects. BAL fluid was obtained at baseline or 24 h after saline challenge, and after the allergen challenges with either surfactant treatment or saline treatment. Values are given as median values and quartiles (in parentheses). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with baseline values and † and ‡ indicate $p < 0.05$ and $p < 0.01$, respectively, compared with saline challenge. For allergen-challenged segments treated with surfactant, # and ## indicate $p < 0.05$ and $p < 0.01$, respectively, compared to saline treatment.

Our finding of increased segmental NO levels following allergen challenge is in line with data obtained 48 h after challenge [19]. Furthermore, the fact that surfactant plus allergen significantly raised segmental NO concentration compared to baseline or sham, whereas surfactant alone had no effect, is in parallel with our recently presented data, indicating that natural porcine surfactant had no effect on its own on inflammation but augmented eosinophilic allergen response [25]. The possibility that the asthmatics in our study developed allergy against Curosurf is rather theoretical since there was no sensitization to this substance and the preparation was given only once. However, Curosurf is a porcine surfactant preparation that might contain pro-inflammatory ingredients, e.g., platelet-activating factor (PAF) [28]. Rather than being an allergen itself, these ingredients might enhance the ongoing eosinophilic inflammation.

At baseline, i.e., before instillation or lavage, there were no differences in NO levels between lung segments, indicating that the observed effects did not result from basal segmental NO variations. There were changes in cellular composition after saline challenge, which we interpreted as a non-specific response. As shown here, the saline challenge led to an increase of the segmental NO values which might indicate that NO is also increased during non-specific inflammation. In addition, effects of saline have to be considered when challenges or treatments are performed with saline solutions as solvent for allergen as in our study. Interestingly, the effect on local NO levels after allergen challenge plus saline was not different from effects of saline challenge alone. Only the stronger stimulus with surfactant plus allergen challenge further increased local NO levels. These data indicate that saline challenge has a significant influence on local NO levels. Therefore, a saline control challenge has to be included when saline is used as a solvent for challenge substances. This aspect has to be considered in the interpretation of previous studies [12,19].

A local saline challenge as performed on day 1 might not only alter segmental NO levels but also total exhaled NO levels. In addition, the bronchoalveolar lavage procedure per se might lead to transient alterations of mucosal transport characteristics as observed after hypertonic saline inhalation [29]. The fact that segmental and exhaled NO were correlated underlined the sensitivity of exhaled NO measurements and the plausibility of local measurements. However, as exhaled NO is integrated over the whole lung, it is impossible to distinguish between treatments in different segments. Therefore, irrespective of the changes observed, measurement of exhaled NO is not suitable to assess local treatment effects in studies with a similar design as our study. Instead, when multiple challenges or treatments are performed in the same patient at the same time, local measurements are indispensable to discriminate between segments that were treated differently.

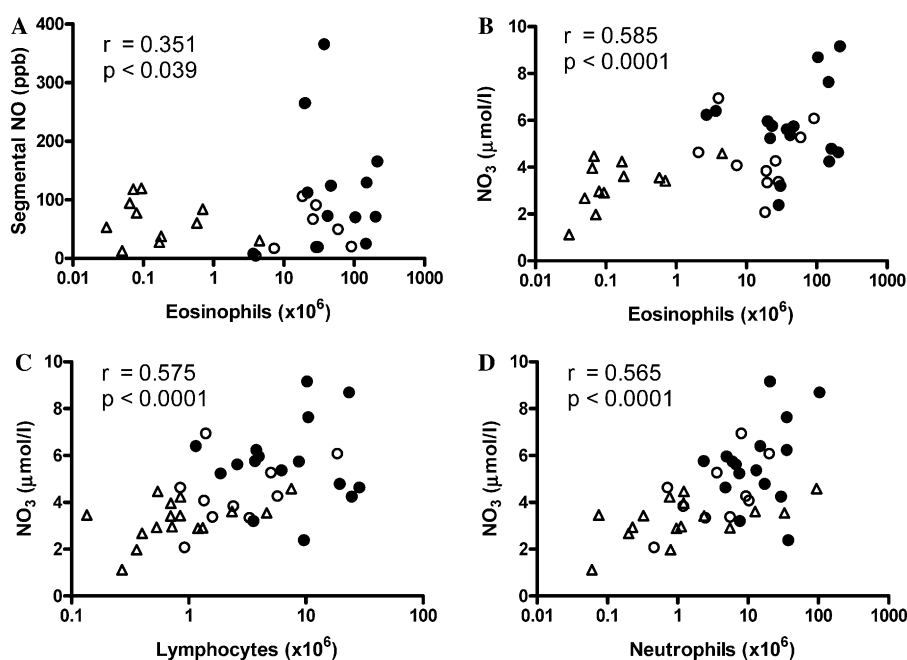


Fig. 4. Correlation of segmental NO levels with eosinophil numbers (A), and correlation of BAL nitrate levels with eosinophil numbers (B), lymphocyte numbers (C), and neutrophil numbers (D) on day 2 after the challenges. Data from segments challenged with saline (open triangles) or allergen (open circles) or allergen plus surfactant (closed circles) are given.

Besides other chemical pathways, NO can react with O_2 to form nitrite and nitrates, and with superoxide anion (O_2^-) to form the highly reactive peroxynitrite ($ONOO^-$). To account for this, we measured nitrite and nitrate concentrations in BAL fluid from the various lung segments. In accordance with previous findings [19], nitrate levels were increased in the asthmatic subjects compared to healthy controls at baseline and further raised 24 h after segmental allergen challenge. Surfactant treatment prior to allergen challenge further raised nitrate levels compared to allergen challenge, indicating the augmentation of airway inflammation.

Both segmental NO levels and BAL nitrate reflected BAL eosinophil numbers. However, differences between allergen challenge with surfactant treatment and allergen challenge without surfactant treatment were only observable by nitrate measurement. Furthermore, the correlation with absolute eosinophil numbers in BAL fluid was stronger for nitrate than for segmental NO, and only nitrate BAL levels correlated with neutrophil and lymphocyte numbers after the challenges. Therefore, nitrate seemed to be more sensitive to local inflammation than segmental NO, possibly because it reflected not only the fraction of NO released into the airways but also additional sources. It is known that eosinophil peroxidase nitrates protein tyrosyl residues [30]. Since there is a large leakage of proteins into BAL fluid after allergen challenge, an increased amount of NO might be transformed and bound to proteins in the presence of eosinophils. NO released from such proteins during the chemiluminescence analysis might have substantially

contributed to the increased level of total nitrate detected. In addition, BAL recovers fluid from small airways and lung periphery both of which are targeted by the instilled solution, whereas gaseous NO produced in the alveoli is likely to be locally absorbed and therefore virtually undetectable in the exhaled air. This seems to imply that the threshold of inflammatory response which is required to lead to detectable signals is higher for segmental NO than BAL nitrate, as indicated by the weak segmental NO response after instillation of allergen alone compared to allergen plus surfactant.

In accordance with recommendations, exhaled NO was measured as plateau level at a flow of 50 ml/s. There is no standard procedure for measuring intrabronchial NO. We thus adopted an ad hoc approach that was considered adequate and feasible under the conditions given. Theoretically, a predefined expiratory flow during endoscopic measurements, if feasible, would seem to achieve comparability with the determination of exhaled NO. However, segmental flows are unknown and may change when ventilation is reduced after a challenge. If this happens, NO levels would probably increase, as a consequence of a longer bronchial transit time due to reduced flow velocity. It would be impossible to decide whether an increase in segmental NO level was due to inflammation or obstruction. To assess the presence of obstruction by CT [31] or regional pressure-flow measurements [32] does not seem helpful as there is no validated algorithm for correcting NO levels. The application of NO transport models (see, e.g. [33]) would require specification of parameters for which there are

no reliable estimates under the various conditions. Though these models could additionally yield estimates of alveolar NO, the required local high precision measurements at defined local flow rates do not seem feasible. Based on these considerations, we performed NO measurements during 30 s periods of breath-holding, with the catheter held free in the airway lumen and the bronchoscope in near-wedge position. This implied measuring at the sampling rate of the gas analyzer of about 4 ml/s, regardless of changes in segmental ventilation. During 30 s, a volume of about 120 ml was sampled within the selected segment. Assuming a lung volume of about 5 L distributed over 19 segments, a tight wedge position would have implied an average reduction of segmental volume by 40–50%, which would certainly have led to intra-segmental redistribution of ventilation. Closure of some dependent airways would cause increased flow velocity in others, with concomitant lowering of NO levels. A similar argument would apply if ventilation within a segment would be redistributed after a challenge, and the redistribution effect would counteract a potential increase of NO level due to inflammation. In fact, preliminary experiments showed that measurements in tight wedge position were neither technically feasible nor did they lead to stable readings.

In contrast, a near-wedge position always led to NO plateau readings after about 15–20 s, indicating constant sampling conditions. Near-wedge position meant that some of the air was drawn from distal and some from proximal airways or neighboring segments. One might argue that this has led to ill-defined sampling conditions. As the fluid used for challenge was instilled distally of the tip of the bronchoscope, there were probably no inflammatory processes affecting the proximally drawn NO. Despite this, the observation that NO levels were consistently increased after allergen challenge and changes were in parallel to those of eosinophil numbers suggests that a sufficient amount of air was drawn from inflamed distal airways. Obviously, the bias from proximal air sampling was not capable of masking the NO response to allergic inflammation. There was also a close correlation between segmental and exhaled NO values before and after the challenges. These findings support the assertion that the method of segmental NO measurement, despite being a technical compromise, was valid in detecting elevated local NO production. The variability observed was probably not due to methodological factors alone but also to variability of biological responses, as known from measurements of exhaled NO.

Compared to baseline, exhaled NO levels decreased 4 h after allergen challenge in the subjects with asthma. Recent studies also reported a tendency toward reduced NO levels early after allergen provocation [19,34], possibly due to airway obstruction resulting in impaired transport of NO into the airway lumen. In contrast, exhaled NO concentration was elevated 24 h

after segmental allergen challenge, in line with data obtained 21 and 48 h after allergen inhalation challenge [11,34]. These findings indicate that the segmental allergen challenge is a strong stimulus with regard to inflammation, and that challenge of only two segments can be sufficient to cause an increase in exhaled NO level in subjects with asthma.

In conclusion, we found that segmental NO levels measured online during bronchoscopy were correlated with exhaled NO levels in subjects with mild asthma, both at baseline and after segmental allergen challenge. Both segmental NO levels and nitrate levels showed an association with regional inflammation in terms of eosinophil numbers but the augmentation of allergen responses by surfactant as demonstrated for eosinophils was better reflected by nitrate levels. In studies using the segmental provocation model, segmental NO and nitrate measurements could be suitable for local monitoring of allergic airway inflammation. Though less sensitive than BAL nitrate, the measurement of segmental NO might be of particular interest when studying parallel or sequential responses in a number of lung segments, as it does not interfere with local inflammation.

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