

Inducible nitric oxide synthase expression is increased in the alveolar compartment of asthmatic patients

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Abstract

Introduction: Increased exhaled nitric oxide (NO) levels in asthma are suggested to be through inducible NO synthase (iNOS). The aim of this study was to investigate the expression of iNOS in bronchoalveolar lavage (BAL) cells and tissue from central and peripheral airways and compare it with the exhaled bronchial and alveolar NO levels in patients with asthma vs a control group.

Methods: Thirty-two patients with asthma (defined as controlled or uncontrolled according to Asthma Control Test score cut-off: 20) and eight healthy controls were included. Exhaled NO was measured, and alveolar concentration and bronchial flux were calculated. iNOS was measured in central and peripheral lung biopsies, as well as BAL cells. Bronchoalveolar lavage macrophages were stimulated *in vitro*, and iNOS expression and NO production were investigated.

Results: Expression of iNOS was increased in central airway tissue and the alveolar compartment in uncontrolled as compared to controlled asthmatics and healthy controls. There were no differences, however, in iNOS mRNA levels in total BAL cells in uncontrolled as compared to controlled asthma. Bronchoalveolar lavage cell mRNA levels of iNOS or iNOS expression in central and alveolar tissue did not relate to alveolar NO, nor to bronchial flux of NO. *In vitro* stimulation with leukotriene D₄ increased iNOS mRNA levels and NO production in cultured BAL macrophages.

Conclusion: The levels of both bronchial and alveolar iNOS are increased in uncontrolled as compared to controlled asthma. However, levels of iNOS in BAL macrophages were not reflected by alveolar NO. Both central and distal iNOS levels may reflect responsiveness to steroid treatment.

The inflammatory mediator nitric oxide (NO) has been shown to be increased in exhaled breath in patients with asthma (1). Patients with high exhaled NO levels have a higher degree of inflammation and more airway reactivity (2). Exhaled NO may be further subdivided into alveolar NO concentration and bronchial flux of NO (3), both of which are shown to be higher in allergic rhinitis patients with asthma compared to both healthy subjects and patients with allergic rhinitis only (4). In patients with asthma who are not responding well to treatment, alveolar NO levels are not reduced by treatment with inhaled corticosteroids (ICS) but are decreased with oral corticosteroid treatment (5). This supports the idea that NO synthesis and inflammatory processes are localized in the alveoli in uncontrolled asthmatics, and less affected by ICS (5). However, the deposition of ICS

particles in the alveolar compartment depends on the inhalation device used, patient performance, etc.

The synthesis of NO is catalysed by the enzyme NO synthase (NOS), of which all the three isoenzymes, inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS), are expressed in the respiratory system (6). The main factor underlying this increased NO level is most certainly iNOS (7), which has been shown to be upregulated in the respiratory epithelium of patients with asthma (8–10). Inducible NOS is primarily located in the apical part of the respiratory epithelium in the lower airways (10–12), and there is a direct link between iNOS and NO gas release (13).

Different haplotypes in the iNOS gene are associated with exhaled NO (14), and polymorphisms in the iNOS gene influence exhaled NO levels (15, 16). Cells from bronchial

epithelial brushings show increased iNOS expression in asthma, especially in severe asthma, as well as a correlation with exhaled NO (7, 17). In contrast, there is no correlation between iNOS in bronchial tissue and exhaled NO (18).

Even though iNOS is mainly expressed in airway epithelial cells, it has also been found in inflammatory cells, such as macrophages (19) and eosinophils (20). There is also evidence for the occurrence of iNOS protein expression and NO production in human macrophages cultured *in vitro* (21).

The increased expression of iNOS in asthma may be a response to locally higher levels of inflammatory cytokines. The role of the Th2 cytokines interleukin (IL)-4 and IL-13 on iNOS involvement is evident. It has, for example, been shown that IL-13, alone, is able to induce iNOS expression in human primary epithelial cells (13, 22), as well as induce a concomitant NO gas release (13). In addition, targeting recombinant IL-4 receptor or IL-4 reduced exhaled NO in patients with atopic asthma (23, 24). Also leukotriene D₄ (LTD₄) has been shown to be linked to iNOS. NO synthase inhibitors have been shown to inhibit the effect of cysteinyl leukotrienes (25, 26), and also specifically that the LTD₄-induced effect was mediated by NO (25).

The expression of iNOS in the bronchial compartment and its relation to exhaled NO is well known, but its relevance in the alveolar compartment and its relation to alveolar NO are still unknown. The aim of this study was to investigate the expression of iNOS in bronchoalveolar lavage (BAL) cells and in tissue from both bronchial airways and alveolar parenchyma and compare it to the respective bronchial and alveolar exhaled NO levels in patients with asthma and healthy controls. Furthermore, we aimed to investigate the stimulatory effect of inflammatory biomarkers on iNOS and NO production.

Methods

Patients

Forty-six subjects were included in this study, 38 patients with asthma and eight healthy controls (Table 1). All patients with

asthma had mild/moderate asthma according to GINA guidelines (27) and reported weekly respiratory symptoms. The asthmatic subjects completed the Asthma Control Test (ACT).

The eight healthy controls had no atopy (negative skin prick test) or respiratory symptoms and a negative methacholine test.

All subjects were nonsmokers and free of respiratory tract infection for at least 3 weeks prior to investigation. All investigations were performed out of pollen season. Bronchial hyper-responsiveness was measured through a methacholine challenge test performed as previously described (28) (see Appendix S1).

The study was approved by the local ethics committee in Lund (LU-412/03), and all subjects gave written informed consent.

NO measurement

A NIOX Flex (Aerocrine AB, Stockholm, Sweden) was used to measure exhaled NO at flow rates of 50 (giving FENO₅₀), 100, 200 and 300 ml/s, 3–4 times/each. Estimations of alveolar NO concentration and bronchial NO flux were made in a two-compartment linear model (3) where flow rates of 100–300 ml/s were used.

Bronchoscopy

Bronchoscopy was performed with local anaesthetics through clinical standard routine. A flexible bronchoscope (Olympus IT60, Tokyo, Japan) with a working channel of 2.6 mm was used together with a biopsy forceps (Olympus FB211D).

Bronchial and transbronchial biopsies

Central biopsies were taken in segmental and subsegmental carina of the right upper lobe. Transbronchial biopsies were performed by means of biopsy forceps guided by fluoroscopy in the right lower lobe. Tissues for the transbronchial biopsies were taken from the periphery at a minimum of 2 cm from the chest wall, to avoid a pneumothorax.

Table 1 Patient characteristics

	Healthy controls <i>n</i> = 8	Controlled asthmatics <i>n</i> = 15	Uncontrolled asthmatics <i>n</i> = 23
Female/male	5/3	7/8	9/14
Age, years	23 (22–28)	29 (24–34)	38 (27–50)
Atopy, <i>n</i>	0	11	22
Perennial allergy, <i>n</i>	0	11	16
Seasonal allergy, <i>n</i>	0	0	5
ACT score	n.a.	23 (21–24)	17 (12–19)
FEV ₁ , l	3.7 (3.3–5.1)	4.0 (3.3–4.6)	3.4 (2.5–3.9)
FEV ₁ , %p	98 (91–113)	92 (86–99)	82 (73–92)
PD20	<2000	1100 (347–2000)*	303 (118–531)†
Positive methacholine, <i>n</i>	0	9*	19†
ICS (µg daily dose)	n.a.	400 (200–800)	800 (400–800)

ACT, asthma control test; PD20, provocative dose of methacholine giving rise to a fall in FEV₁ of >20%; ICS, inhaled corticosteroids (equivalent dose to budesonide); n.a., not applicable.

* and † = 2 and 3 patients, respectively, excluded from methacholine provocation test due to FEV₁ < 70%p.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed with 120–150 ml (3×50 ml) of 0.9% phosphate-buffered saline (PBS). The volume of the recovered lavage fluid was a median of 86 ml (IQR: 75–90 ml). The BAL was filtered through a 100- μ m filter and centrifuged at 200 *g* for 5 min (+4°C). The BAL cells were thereafter resuspended in PBS, and one fraction of the cells was subjected to cytopspin for differential counting after staining with May–Grunwald–Giemsa stain. Another fraction of the BAL cells was lysed for RNA preparation of total BAL cell mRNA, and the last fraction was plated for *in vitro* stimulation experiments (depending on BAL cell availability).

Tissue fixation and immunohistochemical staining

The biopsies were placed in a 4% buffered formaldehyde, dehydrated and embedded in paraffin for storage. Three-micrometre-thick sections were then made using a microtome and stained with Mayer's haematoxylin. Thereafter, the tissues were examined histologically at low-power magnification to select biopsies that had a well-preserved overall morphology. The sections were blinded, and based on the morphological criteria only, two bronchial and two transbronchial biopsies were chosen from each patient.

After antigen retrieval, immunohistochemistry for iNOS was performed with the Dako REAL™ EnVision™ Detection System using a mouse monoclonal anti-human iNOS (R&D Systems, Abingdon, UK) (see Appendix S1).

Quantification of iNOS and histological analysis

The stained slides were scanned using an automated slide scanner with a 20 $\times$ microscope lens (ScanScope CSTM; Aperio, Vista, CA, USA), and high-resolution and digital images were produced. The images were analysed via the image analysing program vis (Visiomorph™, Visiopharm Integrator System, Visiopharm, Hoersholm, Denmark), and settings were made to detect DAB staining for iNOS as well as the total tissue area (excluding air) in μm^2 . Large vessels, glands and blood were excluded from the analysis. The expression of DAB per tissue area (μm^2) was calculated in two bronchial and transbronchial biopsies, and a respective average for each patient was calculated.

Plating and *in vitro* stimulation of BAL macrophages

Bronchoalveolar lavage cells were plated (350 000–500 000 cells/well depending on BAL cell availability), and the day after plating, all nonadherent BAL cells were rinsed away (for cell culture details, see Appendix S1).

Adherent BAL cells were stimulated for 24 h in the same medium (lacking phenol red due to its interference with the nitrite and nitrate assay) with IL-4 (10 mg/ml), IL-5 (10 mg/ml), IL-13 (10 mg/ml), the three in combination, or LTD₄ (10^{-7} M). Medium was collected for analysis of total nitrite and nitrate concentration, and cells were lysed for RNA preparation and PCR.

BAL cell fixation and immunocytochemical staining

To investigate iNOS and CD68 staining in total BAL cells as well as *in vitro* cultured (adherent) BAL macrophages, the cells were stained for iNOS and CD68 (clone PG-M1, monoclonal mouse anti-human IgG3; DAKO, Glostrup, Denmark). For a detailed description, see Appendix S1.

Semi-quantitative real-time PCR

RNA preparation was performed followed by cDNA synthesis and semi-quantitative real-time PCR. Beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used as housekeeping genes. For primer sequences, see Appendix S1. The cycle threshold (C_t) was used to determine the mRNA expression relative to housekeeping genes (see Appendix S1).

Measurement of NO synthesis

Nitric oxide synthesis was measured indirectly by the amount of total nitrate and nitrite in the cell medium using a chemiluminescence assay from Cayman Chemicals, Ann Arbor, MI, USA (780001). The assay is based on a Griess reaction, and analyses were performed according to the manufacturer's instructions. In short, the kit has been used to measure total nitrate and nitrite concentration in a simple two-step process. The first step was the conversion of nitrate to nitrite utilizing nitrate reductase. The second step was the addition of the Griess reagents which convert nitrite into a deep purple azo compound, which is measured photometrically and estimates nitrite concentration according to a nitrite standard.

Statistical analysis

GRAPHPAD PRISM version 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for data analyses, and the results are shown as a median (interquartile range). Kruskal–Wallis test was used for comparison between several groups, with subsequent Dunn's multiple-comparison test for comparison among the separate groups. Mann–Whitney rank-sum test was used for comparison between two separate groups, Wilcoxon test was used for paired data and Spearman's correlation test was used for correlation analyses. A *P*-value ≥ 0.05 was used for a statistically significant result.

Results

Subjects

Fifteen patients were defined as controlled asthmatics, that is having an ACT score ≥ 20 , and 23 patients had uncontrolled asthma (ACT < 20), despite treatment with ICS at a dose of 400–800 μg budesonide (or equivalent). Most asthmatic subjects had atopy and a positive methacholine provocation test (defined as PD₂₀-FEV₁ < 2000 μg) (Table 1).

Exhaled NO

All subjects were able to perform successful measurements at all flows. The median of the correlation coefficients of the linearity in the linear model was 0.99 (IQR: 0.93–1.00), and only four subjects showed a correlation coefficient <0.8.

Uncontrolled asthmatics had a significantly higher FENO₅₀ (Fig. 1A) and bronchial flux of NO (Fig. 1B) compared to healthy controls. However, the alveolar NO concentration did not significantly differ between the groups ($P = 0.062$; Fig. 1C).

iNOS expression in tissue

Biopsies from both bronchial and alveolar compartments were obtained from eight healthy controls, eight controlled asthmatics and ten uncontrolled asthmatics. Tissues from bronchial and alveolar compartments both stained positively for iNOS. In bronchial tissue, the staining for iNOS was most abundant in the epithelial layer (Fig. 2A). In tissue from the alveolar compartment, iNOS staining was seen mainly in cells in the alveolar lumen (Fig. 2B). The cells in the alveolar lumen were identified as macrophages by to costaining of CD68 and iNOS (Fig. 2C). Quantification of iNOS immunoreactivity showed a difference in expression among the study groups, both in the bronchial (Fig. 2D,E) and in the alveolar compartment (Fig. 2F). In the bronchial compartment, higher levels of both epithelial and subepithelial iNOS expression were observed in uncontrolled asthmatics compared to controlled asthmatics.

When analysing all subjects, the iNOS expression in the bronchial tissue (epithelial or subepithelial) did not correlate with FENO₅₀, nor the bronchial flux of NO. Neither did the iNOS expression in the alveolar compartment correlate with alveolar NO concentration. There were no correlations within the respective patient groups.

iNOS expression in BAL cells

Bronchoalveolar lavage was performed in the asthmatic subjects, and BAL cells from 14 controlled and 16 uncontrolled

asthmatic subjects were investigated. Inducible NOS was present in the BAL cells, and costaining of iNOS and CD68 (Fig. 3A) confirmed that macrophages are the principal cells expressing iNOS in the airway lumen. All macrophages stained positive for iNOS, but with different intensity, suggesting different iNOS levels within the population.

The iNOS expression in the total BAL cell fraction was measured based on mRNA levels. A similar iNOS mRNA expression was seen in total BAL cell fraction from controlled and uncontrolled asthmatics (Fig. 3B). No significant correlation could be seen between alveolar NO concentration and iNOS mRNA levels in BAL cells in all subjects (Fig. 3C), and not within the separate groups either [controlled asthmatics ($P = 0.58$, $r = 0.16$) or uncontrolled asthmatics ($P = 0.19$, $r = 0.33$)].

The proportion of macrophages among the BAL cells was 85 (79–91)% in controlled asthmatics and 92 (89–94)% in uncontrolled asthmatics. After correction for macrophage proportion of BAL cells (multiplying the BAL iNOS mRNA content with the percentage of macrophages), there were no differences in BAL iNOS mRNA expression between controlled and uncontrolled asthmatics (Fig. 3D), and no correlation between iNOS mRNA expression and alveolar NO concentration in all subjects (Fig. 3E) or within the separate groups [controlled asthmatics ($P = 0.69$, $r = -0.14$) or uncontrolled asthmatics ($P = 0.18$, $r = 0.34$)].

Stimulation of iNOS and NO production in adherent BAL macrophages

Bronchoalveolar lavage cells were plated, and adherent BAL macrophages were further *in vitro* stimulated. Costaining of iNOS and CD68 evidenced that adherent BAL cells were in fact macrophages (Fig. 4A). There were no significant differences in iNOS mRNA levels in adherent BAL macrophages from controlled and uncontrolled asthmatics (Fig. 4B). An *in vitro* stimulation with LTD₄ increased the mRNA levels of iNOS in the adherent BAL cells ($P = 0.019$) (Fig. 4C). No significant change in mRNA levels after stimulation with IL-4 ($P = 0.98$), IL-5 ($P = 0.25$), IL-13 ($P = 0.25$) or them all in combination together ($P = 0.11$) could be seen.

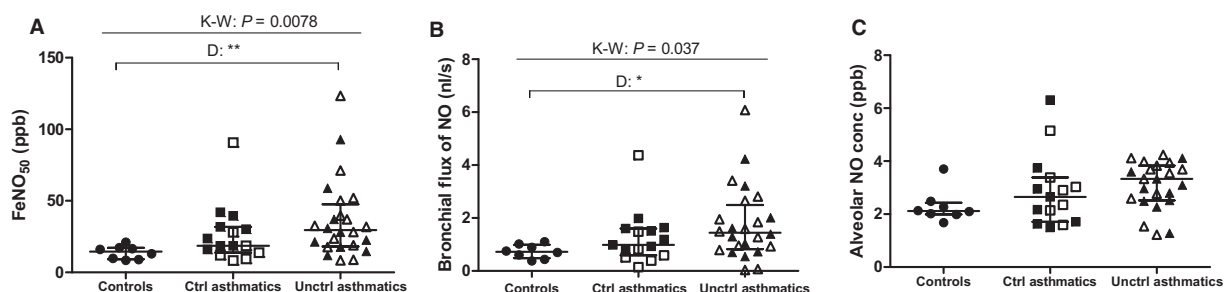


Figure 1 Exhaled nitric oxide (NO) levels shown as FENO₅₀ (A), bronchial flux of NO (B) and alveolar NO concentration (C) in healthy subjects, controlled asthmatics and uncontrolled asthmatics. Ctrl = controlled; Unctrl = uncontrolled. Data are presented as

individual dots and medians. Patients included (filled symbols) and patients not included (open symbols) in biopsy inducible NOS (iNOS) investigation are marked. K-W, Kruskal-Wallis; D, Dunn's. * $p < 0.05$ and ** $p < 0.01$

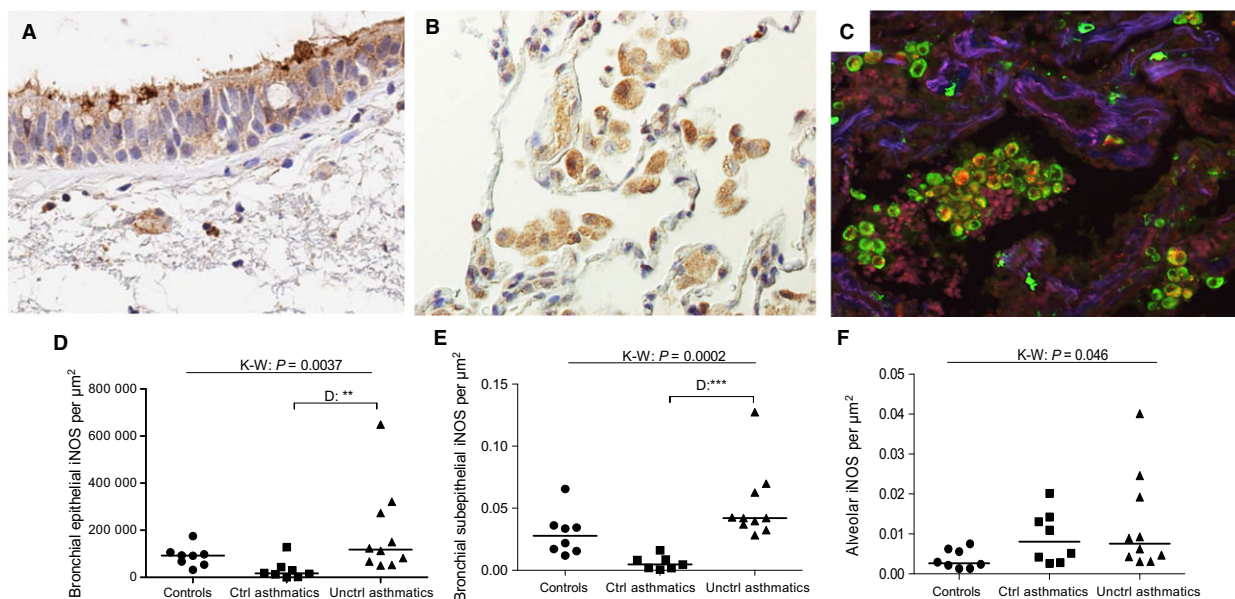


Figure 2 Expression of inducible NOS (iNOS) (brown staining) in central airway tissue (A) and alveolar parenchyma in a transbronchial biopsy from a patient with asthma (B). Double immunofluorescence and costaining of iNOS (red) and CD68 (green) in alveolar tissue reveals alveolar macrophages as the major source for iNOS

expression in this compartment (C). Quantification of iNOS levels in tissue from the bronchial epithelial (D), bronchial subepithelial (E) and alveolar (F) compartment showed increased iNOS expression in uncontrolled as compared to controlled asthmatics and healthy controls. K-W, Kruskal-Wallis; D, Dunn's. ** $p < 0.01$ and *** $p < 0.001$

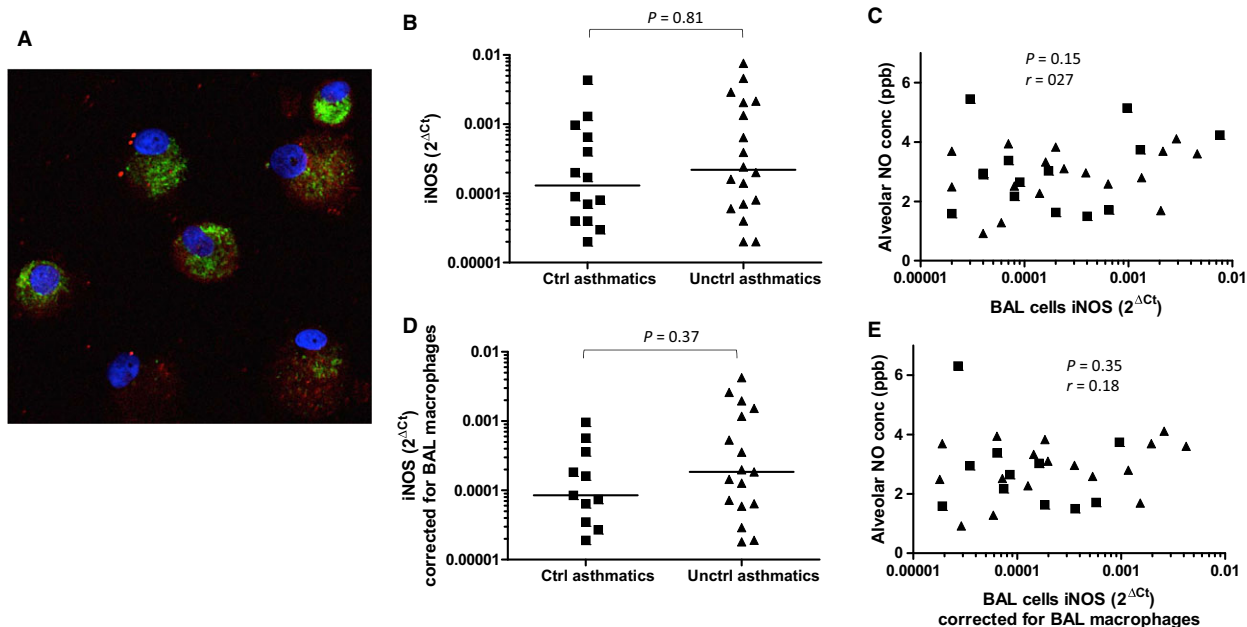


Figure 3 Bronchoalveolar lavage (BAL) cell staining of inducible NOS (iNOS) (red) and CD68 (green) show iNOS staining in the macrophages (A). Levels of iNOS in total BAL cell mRNA did not differ between controlled and uncontrolled asthmatics (B), and no correlation could be seen between alveolar nitric oxide (NO)

concentration and iNOS mRNA levels in total BAL cells (C). As iNOS is expressed in BAL macrophages, the iNOS mRNA levels were corrected for BAL macrophage content (D), but still no correlation could be seen to alveolar NO concentration (E). ■ = controlled asthmatics; ▲ = uncontrolled asthmatics.

To investigate the corresponding NO production, the cell medium from the adherent BAL macrophages was analysed for nitrite and nitrate concentration. No significant

differences in total nitrite and nitrate levels from adherent BAL macrophages from controlled and uncontrolled asthmatics were seen (Fig. 4D). Corresponding to the iNOS

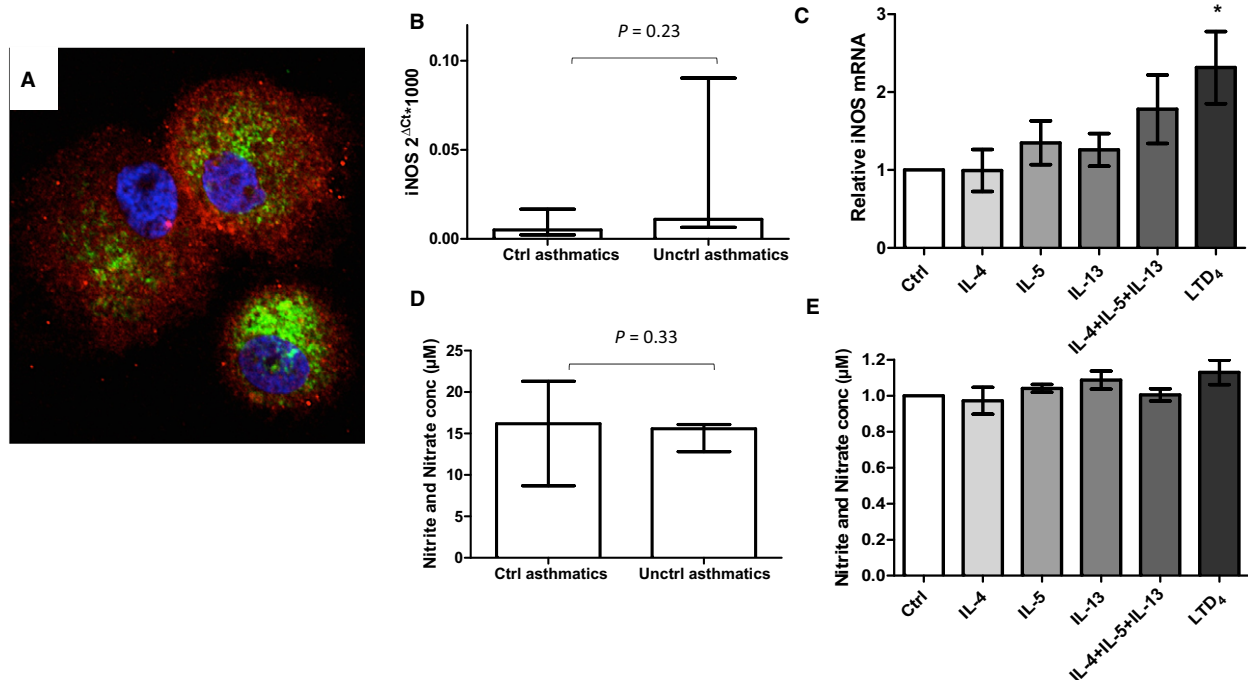


Figure 4 Adherent bronchoalveolar lavage (BAL) macrophages (A) showed staining for inducible NOS (iNOS) (red) and CD68 (green). The iNOS mRNA levels in adherent BAL macrophages from controlled ($n = 7$) and uncontrolled ($n = 7$) asthmatics were measured (B), and also after *in vitro* stimulation for 24 h with IL-4, IL-5 or IL-13 (separately or all in combination) or with LTD₄ (C). In parallel, cell medium from the adherent BAL macrophages from controlled and uncontrolled asthmatics was analysed for total nitrite and nitrate concentration, reflecting nitric oxide (NO) production (D). Nitrite and nitrate concentrations were also measured after stimulation with IL-4, IL-5 or IL-13 (separately or all in combination) or with LTD₄ (E). * $P \leq 0.05$ for relative increase compared to unstimulated controls.

mRNA levels, a trend of a similar pattern in nitrite and nitrate levels in medium from the adherent BAL cells was seen after an *in vitro* stimulation with LTD₄, IL-5 and IL-13 (Fig. 4E), however not significant ($P = 0.079$, $P = 0.078$ and $P = 0.11$, respectively).

Discussion

In this study, we have shown that the expression of iNOS was increased in central airway tissue and alveolar compartment in uncontrolled asthmatics as compared to controlled asthmatics and healthy controls. However, this appears to be tissue-specific and could not be demonstrated in mRNA levels from total BAL cells. In addition, total BAL cell mRNA levels of iNOS or iNOS protein expression in central and alveolar tissue did not relate to alveolar NO, nor to bronchial flux of NO. We also demonstrate that the BAL macrophages are the main iNOS-expressing cells in the alveolar compartment, and *in vitro* stimulation with LTD₄ increased iNOS mRNA levels and NO production in cultured BAL macrophages.

Exhaled NO, both the bronchial and alveolar fraction, is known to be increased in patients with asthma (4), and a decrease in NO is often seen in response to steroid treatment (29). In our study, all subjects were on ICS treatment, which

possibly suppressed NO levels that would otherwise be higher. Exhaled NO levels were higher, however, in patients with asthma than in healthy subjects, and specifically in the uncontrolled asthmatics compared to controlled asthmatics (Fig. 1). This could reflect nonresponsiveness to steroid treatment and involvement in the inflammatory patterns that give rise to the uncontrolled pathophysiological state. In fact, uncontrolled asthmatics had a higher daily dose of ICS compared to controlled asthmatics, but they still exhibited higher levels of exhaled NO.

The level of iNOS has previously been shown to be upregulated in the respiratory epithelium of patients with asthma (8–10), which we also showed in uncontrolled asthmatics in this study. In addition, we also observed an increase in iNOS expression in the alveolar compartment (Fig. 2). Staining of iNOS was seen in cells residing in the alveolar parenchyma, but in addition mainly in macrophages in the alveolar lumen. Uncontrolled asthmatics, but also controlled asthmatics, showed higher levels of iNOS compared to healthy controls, which suggest an increased peripheral airway inflammation. This is in line with the alveolar Th2-skewed inflammation in uncontrolled asthmatics that we have previously shown in these patients (30).

In the bronchial airway tissue, the iNOS levels were suppressed in the controlled asthmatics, while the uncontrolled

asthmatics showed higher levels compared to controls. This may reflect responsiveness to ICS in the controlled asthmatics in contrast to the uncontrolled asthmatics. In contrast, ICS is known to not reach the peripheral airways (5). This may explain why the alveolar compartment was lacking the pattern of iNOS expression seen in the bronchial airways, but rather an overall increase in iNOS expression in the alveolar compartment was observed.

One of our aims was to investigate the main source for the exhaled NO from the alveolar compartment. The bronchial fraction of the exhaled NO has previously been demonstrated to be related to iNOS expression in the bronchial epithelium (7). However, this was not shown in this study, which might be due to differences in methodology, as bronchial brushings were used by Lane et al. (7), compared to bronchial biopsies in the present study. Roos et al. (18) show a correlation between FENO and iNOS expression in the bronchial epithelium, but only after including values both before and after allergen provocation. When they investigated the time points separately, there was in fact now correlation between FENO and bronchial iNOS expression which corresponds to what we see in this study. In contrast, the origin of the alveolar NO is not previously investigated. Our hypothesis was that either iNOS-producing cells in the alveolar parenchyma or lumen would give rise to the increased alveolar NO concentration in patients with asthma. However, no correlation between tissue or BAL cell iNOS levels and alveolar NO concentration could be confirmed. There could be several reasons for this, and one of which is that we have still not found the specific cells responsible for the NO production. We have, in this study, only investigated the overall tissue in the alveolar compartment as well as total BAL cells and BAL macrophages. A less likely reason is that the alveolar NO production is not catalysed by iNOS, but by one of the other NOS (i.e. eNOS or nNOS), although it seems unlikely that eNOS or nNOS would contribute substantially to the exhaled NO. Endothelial NOS has been shown to be produced in much lower levels than iNOS in the bronchi, and nNOS could not be detected at all (7). One should not forget that NO is rapidly absorbed in the alveolar tract, and it may well be that reduced clearance of NO, not increased formation of NO, explains elevated Calv levels in certain conditions, as in the case of ageing individuals (31). Further, NO diffusion from the airways is dependent on the total NO-producing surface area and the respiratory epithelium most probably exceeds the surface area of alveolar macrophages. The periphery of the airways is complex, and the macrophages are contributing to, but are maybe not the major source of, NO in the exhaled breath. A limitation of our study was that we measured only mRNA levels of iNOS, but iNOS is primarily regulated on the transcriptional level (32), so this should not have any significant impact on the results.

Using the linear regression model for estimating the bronchial and alveolar NO fraction is widely discussed. Unfortunately, there is no perfect model that can be used today. For comparison, all our data were also calculated using the

model described by Högman and Merilainen (33) (although that model is only applicable if a lower flow of <30 is used), and similar results were obtained. The model using correction for axial diffusion and trumpet shape of the airways was also applied, but resulted in 15 patients with a negative alveolar NO concentration which is not plausible. However, no correlation between bronchial flux of NO and alveolar NO concentration was found in this study, which suggests that the bronchial flux of NO has no major impact on estimated alveolar NO concentration.

The link between exhaled bronchial NO and iNOS in the bronchial compartment is moreover apparent, as treatment with an iNOS inhibitor reduces iNOS activity in bronchial brush cells, as well as exhaled NO in patients with asthma (34). The link between alveolar NO and iNOS in the alveolar compartment, however, is not that obvious. Inhalation of a nebulized iNOS inhibitor decreased airway wall NO flux, but did not affect alveolar NO concentration or diffusing capacity of NO (35). This might, of course, depend on the less accessible alveolar compartment.

One apparent finding in this study was that macrophages in the alveolar lumen express iNOS, and we therefore further investigated BAL macrophages *in vitro*. It has previously been shown that removal of these cells from *in vivo* to *in vitro* conditions gives a loss of iNOS activity (11), and due to low iNOS levels and limited amount of BAL cells, we could not perform these experiments in all individuals. There were no significant differences between stimulation of BAL macrophages from controlled ($n = 7$) and uncontrolled ($n = 4$) asthmatics, and these groups are therefore presented together in Fig. 4. Regardless, both higher iNOS mRNA level and a subsequent increase in NO production (measured as nitrite and nitrate level) could be seen after stimulation with LTD₄. This demonstrates that LTD₄ is involved in the production of NO. Similarly, the Th2 cytokines, IL-5 and IL-13, showed a tendency towards an increase in NO production. Exhaled NO is known to be related to the Th2-response, and as we hypothesized, these cytokines could possibly be involved in the NO production by a direct stimulation of BAL macrophages.

In conclusion, our study shows that the expression of iNOS is increased in both central airways and the alveolar parenchyma, but not in BAL cells, in uncontrolled asthmatics as compared to controlled asthmatics and healthy controls. Neither iNOS levels in BAL cells nor those in alveolar tissue were related to alveolar NO, suggesting another source of NO. We also show that the BAL macrophages are the main iNOS-expressing cells in the alveolar compartment, and *in vitro* stimulation with LTD₄ gives rise to increased NO production in cultured BAL macrophages.

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Author contributions

ET designed the study, did collection, analysis and interpretation of data and drafted the article. CA and JW did analysis and interpretation of data and revised the article critically. JE did interpretation of data and revised the article critically. LB designed the study, did interpretation of data and revised the article critically. All authors have approved the final version to be published.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. iNOS methodological supplement.

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