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Title page

iNOS affects matrix production in distal lung fibroblasts from patients with mild asthma

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Abbreviations: Extracellular matrix (ECM); inhaled corticosteroid (ICS); nitric oxide (NO); inducible nitric oxide synthase (iNOS); proteoglycans (PG);

ABSTRACT

Introduction: A high level of exhaled nitric oxide (NO) is a marker for inflammation in the airways of asthmatic subjects. However, little is known about how NO and inducible nitric oxides synthase (iNOS) activity may affect remodelling in the distal lung. We hypothesized that there is a link between iNOS and ongoing remodelling processes in the distal lung of mild asthmatics.

Methods: Patients with mild asthma (n=6) and healthy control subjects (n=8) were included. Exhaled NO was measured at different flow rates and alveolar NO concentrations were calculated. For studies of remodelling processes in the distal lung, primary fibroblasts were grown from transbronchial biopsies and stimulated with unselective and selective NOS inhibitors or a NO donor. The mRNA expression of iNOS and synthesis of NO (indirectly as nitrite/nitrate) were measured and distal lung fibroblast synthesis of the extracellular matrix proteoglycans were analysed.

Results: The distal lung fibroblasts expressed iNOS, and there was a tendency of higher expression in fibroblasts from patients with asthma. The selective iNOS inhibitor 1400W inhibited iNOS expression and NO synthesis in fibroblasts from patients with asthma (p=0.031). Treatment with 1400W significantly increased synthesis of the proteoglycan versican (p=0.018) in distal fibroblasts from patients with asthma whereas there were no effects in fibroblasts from control subjects.

Conclusions: Our data suggest that there is a link between iNOS and remodelling in the distal lung of subjects with mild asthma and that iNOS could have a modulatory role in pathological airway remodelling.

Keywords: asthma, inducible nitric oxide synthase, lung fibroblast, proteoglycan, remodelling

1. INTRODUCTION

In recent years the view of asthma as an inflammatory central airway disease mainly involving inflammatory cells has changed (1). It is now becoming evident that also the distal airways are subjected to inflammatory and structural changes (2, 3). In addition to the chronic inflammation associated with asthma, there are multiple structural alterations in the lung tissue, a phenomenon collectively termed airway remodelling. Remodelling and inflammation in the subepithelial compartment in central airways of asthmatic subjects is now quite well characterized (4) and there is now a strong consensus that airway remodelling contributes to the decline in lung function in patients with asthma (5, 6). However, less is known about the onset and cause of remodelling in distal airways of asthmatic subjects. Currently, exhaled NO is used as a non-invasive marker for airway inflammation and an increased production of NO is seen in the exhaled air of patients with asthma (7, 8). Several studies have been published suggesting different modulatory roles of NO in asthma. Though, depending on site as well as the amount generated, NO may exert both beneficial and harmful effects in the airways (9). There are three different isoforms of the nitric oxide synthase (NOS) that generates NO from the amino acid L-arginine; neuronal NOS (nNOS/NOS-1), inducible NOS (iNOS/NOS-2) and endothelial NOS (eNOS/NOS-3) (10). iNOS generates considerably larger amounts of NO than the constitutively expressed nNOS and eNOS, which has caused speculation that the large amounts of NO generated by iNOS in bronchial epithelial cells may amplify the inflammatory response in asthma (10, 11). Inhaled corticosteroids are known to reduce NO synthesis in the airways (7, 12) and exhaled NO may be used to adjust ICS therapy (13). Importantly, new insights are emerging regarding differential roles of NO in inflammation and fibrosis (14). However, less is known regarding the role of more distally produced NO and the effect on remodelling processes and extracellular matrix (ECM) in the peripheral lung. As primary ECM producing cells, the lung fibroblasts are key players in remodelling

processes by depositing ECM molecules such as proteoglycans (15, 16). Together these ECM molecules play a crucial role in maintaining tissue integrity and regulating tissue inflammation by storing cytokines and growth factors, which can be released under certain circumstances (17). In mild asthma we recently demonstrated the presence of remodelling in distal airways as shown by increased collagen content in the tissue. In addition a negative correlation was found between alveolar NO and the production of the proteoglycans biglycan and decorin by fibroblasts from patients with mild asthma (2). We therefore hypothesized that there is an ongoing remodelling that is linked to iNOS in the distal airways of patients with mild asthma. To test this hypothesis we used distal lung fibroblasts derived from transbronchial biopsies from patients with mild asthma and healthy control subjects. Our results show that iNOS is linked to distal airway remodelling and ECM synthesis which implies a modulatory role of iNOS in ongoing pathological remodelling processes in the distal lung of asthmatic subjects.

2. METHODS

2.1. Patient characteristics

The current study included patients with mild allergic asthma (n=6) according to Global Initiative for Asthma (GINA) guidelines (18) and healthy non-smoking controls (n=8) with no other airway diseases. The patients with mild asthma included in this study were not treated with corticosteroids. Three out of eight control subjects were lung donors with no former history of lung disease. Written informed consent was obtained from all participants or from the closest relative. The study was approved by the ethic committee in Lund (LU412-03, FEK 213/2005 and FEK 413/2008) (table 1).

Table 1. Patient characteristics of subjects included in the study.

Characteristics	Healthy controls (n=5+3 [#])	Patients with mild asthma (n=6)
Age (years)	23 (23-29)	26 (22-37)
Gender	2M/3F	4M/2F
Atopy (n)	0	6
FEV ₁ (l)	3,7 (3.5-5.4)	4,1 (3.2-6.0)
FEV ₁ (% predicted)	101 (95-116)	95 (88-124)
PD20 (µg methacholine)	>2000	101 (0.05->2000)*

[#] These three subjects were lung donors with no former history of lung disease. Of the lung donors, two out of three had the age interval 46-65 years old and one between 31-45 years old. There were no other parameters available for these three individuals. Table values are therefore presented only from the other five control subjects. FEV₁: Forced expiratory volume in 1 second; PD20: Cumulative dose of methacholine that gives a 20% fall in FEV₁.

*=Significant differences (p<0.05) compared to healthy controls. Data are presented as median (range).

2.2. NO measurements

Measurements were performed as previously described (19). Briefly, FeNO measurements were done prior to bronchial challenge testing at a flow rate of 50 (giving FeNO₅₀), 100, 200, 300 and 400 ml/s using a NIOX NO analyser (Aerocrine AB, Stockholm, Sweden), and the results were expressed as parts per billion (ppb). Alveolar NO concentration and bronchial flux of NO were calculated with a two-compartment linear model using a flow rate of 100-400 ml/s (20).

2.3. Collection of transbronchial biopsies and fibroblast cultures

Primary human distal lung fibroblast cultures were established from the distal biopsies from patients with mild asthma (n=6) and healthy control subjects (n=5) as previously described (2). Some control lung fibroblasts (n=3) were instead isolated from lung explants from unused donors meant for lung transplantation (21). Importantly, alveolar parenchymal specimens from lung explants were collected 2-3 cm from the pleura in the lower lobes, equivalent to the location where the transbronchial biopsies were obtained. Vessels and small airways were removed from the peripheral lung tissues. From biopsies and explant samples of similar size, cultures of primary fibroblast-like cells were established. Briefly, transbronchial biopsies and parenchymal specimens were transferred to cell culture medium immediately after sampling. Parenchymal pieces from biopsies and parenchymal specimens were cut into small pieces that were allowed to adhere to the plastic of cell culture flasks for 4 h and were then kept in cell culture medium in 37°C cell incubators until there were outgrowths of cells with morphology typical for fibroblasts. Primary fibroblasts were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal Clone III serum (FCIII, Hyclone, Logan, UT, US), 1% L-glutamine, 0.5% gentamicin and 5 µg/ml amphotericin B (all from Gibco BRL, Paisley, UK). The fibroblast cultures were stained with specific antibodies to verify the mesenchymal identity and to estimate the purity, as previously described (2, 21). Isolated primary fibroblasts were split 1:2 at expansions and were used in passages 4–7 for further experiments. The mesenchymal identity of the fibroblasts was verified by positive staining for the following markers: vimentin, a member of the intermediate filament family and important for the structural integrity of the fibroblast; alpha-SMA, a protein involved in contractile apparatus; prolyl 4-hydroxylase, an enzyme involved in collagen synthesis, as previously described (2, 21, 22).

2.4. Cell stimulations with NO synthase inhibitors or NO donor

The distal lung fibroblasts (asthma n=6, controls n=8) were treated with the selective iNOS inhibitor N-(3-(Aminomethyl)benzyl)acetamidine (1400W) 1 μ M (23), the unselective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) 30 μ M or the NO donor DETA-NONOate 10 μ M (all from Cayman Chemicals, Ann Arbor, MI). Screenings with different concentrations of 1400W (0.1-1-10 μ M), L-NAME (10-30-100 μ M) and DETA-NONOate (1-10-100 μ M) were performed to find an optimal single dose for stimulations of the primary distal lung fibroblasts. Stimulations were performed in 0.4% FCIII for 24 hours.

2.5. iNOS mRNA expression in distal lung fibroblasts

RNA preparation of the lung fibroblasts was performed on the cell lysate using RNeasy Mini kit (from Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol. cDNA was synthesized using iScript™ cDNA Synthesis Kit from Bio-Rad Laboratories (Hercules, CA). Semi-quantitative real-time PCR was performed on an Applied Biosystem 7900 thermocycler using iTaq™ SYBR Green Supermix with ROX from Bio-Rad Laboratories. Beta-actin was used as housekeeping gene. Primers (from Invitrogen™), were used at 300 nM. The sequences for the iNOS primers were; forward: ACA AGC CTA CCC CTC CAG AT and reverse: TCC CGT CAG TTG GTA GGT TC and b-actin primers were; forward: AGC ACA GAG CCT CGC CTT T and reverse: GGA ATC CTT CTG ACC CAT GC. Amplicon lengths were 158 p and 214 bp, respectively. The cycle threshold (C_t) was determined for each sample, and quantification of the gene expression was assessed with a comparative cycle threshold (C_t) method. The mRNA expression relative to housekeeping genes was determined by subtracting the C_t values for iNOS from the C_t value for the housekeeping gene ($=\Delta C_t$). Data are depicted as $2^{\Delta C_t} \times 10^5$. For calculations of the relative gene expression after treatment,

the Pfaffl method was used, taking both housekeeping gene expression and primer efficiency (Eff) into account, i.e. $\text{Ratio} = (\text{Eff}_{\text{target}}^{\Delta\text{Ct, target(calibrator-test)}}) / (\text{Eff}_{\text{reference}}^{\Delta\text{Ct, reference(calibrator-test)}})$. This method assesses the difference in gene expression between the genes of interest and internal standard housekeeping gene for each sample to generate a ratio between the samples. Beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) have previously been used as housekeeping genes in these fibroblasts from control subjects and patients with asthma (24) and there were no differences in the expression of these genes between patient with asthma and control subjects. Due to limited amount of material, only one housekeeping gene (beta-actin) was used in this material.

2.6. Measurement of NO synthesis in distal lung fibroblasts

Corresponding NO synthesis was measured indirectly by the amount of nitrate/nitrite in the cell medium (without phenol red) using a chemiluminescence analysis from Cayman Chemicals, Ann Arbor, MI (780001). The assay is based on a Griess reaction, and analyses were performed according to the manufacturer's protocol.

2.7. Analysis of proteoglycan production

Proteoglycan production in fibroblasts was determined as previously described (25, 26). Briefly, lung fibroblasts were cultured in 6-well plates. Cells were incubated in low sulphate DMEM (Gibco BRL, Paisley, UK), pretreated with the NOS inhibitors or NO donor in ³⁵sulfate containing DMEM for 24 hours in duplicates. Cell medium with 0.4% serum was used as a control of basal activity. Proteoglycan synthesis was quantified by ³⁵sulfate

incorporation into glycosaminoglycan side-chains measured on a scintillation counter (Wallac; Perkin Ellmer, Boston, MA, US). Individual proteoglycans were separated by ion exchanger DEAE52 and SDS-PAGE and then quantified using densitometry. The various proteoglycans have previously been identified by mass spectrometry (27). Proteoglycan production in the medium was related to the total amount of protein in the corresponding cell layer. The amount of proteins in the cell lysate was analysed by a commercially available protein assay, which constitutes a colorimetric assay with BSA as a standard reference for measuring total protein concentrations (Bio-Rad Laboratories, Hercules, CA, US).

2.8. Statistical analysis

Data are presented as median values together with range. For non-parametric data, between-groups comparisons were performed with the Kruskal–Wallis test or with the Dunn post-hoc multiple comparison test followed by the Mann–Whitney U test. Proteoglycans was analysed by one-sample t-test. P values of <0.05 (*) denote significant levels of difference. All analyses were performed using GraphPad Prism version 6.00 software (GraphPad Software, San Diego USA).

3. RESULTS

3.1. Demographic and clinical characteristic

An overview of patient characteristics is presented in Table 1. There were no significant differences in age or gender. Patients with mild asthma were characterised by their sensitivity to methacholine provocation compared to the healthy control subjects ($p=0.017$). Patients with asthma presented a tendency of higher FeNO_{50} values ($p=0.082$), but we could not detect

any significant differences of higher bronchial flux of NO ($p=0.12$) and alveolar NO concentration ($p=0.14$) compared to healthy controls (figure 1.A-C).

3.2. iNOS expression and NO synthesis in pulmonary fibroblasts

The primary distal lung fibroblasts expressed iNOS mRNA (fig 2A). However, we could not detect any significant differences of iNOS mRNA expression between distal lung fibroblasts from patients with asthma and control subjects ($p=0.14$, fig 2A) neither did treatments with NOS inhibitors or NO donor affect iNOS mRNA expression (see supplement 1A and B). NO synthesis, measured as nitrite/nitrate concentration in cell medium after stimulation (fig 2B), was significantly reduced by selective iNOS inhibition with 1400W in fibroblasts from subjects with mild asthma ($p=0.031$, fig 2C), whereas there was no effect of iNOS inhibition in fibroblasts from control subjects (fig 2D). Either the unselective NOS inhibitor or the NO donor significantly affected NO synthesis in distal lung fibroblasts from patients with mild asthma or control subjects (fig 2 C and D).

3.3. Nitric oxide and its relation to proteoglycan production

In general, distal lung fibroblasts from control subjects did not respond to either NOS inhibition or NO stimulation (fig 3B, D, F, H). In contrast, 1 μ M of 1400W significantly increased versican production in fibroblasts from patients with mild asthma compared to controls ($p=0.018$; Fig 3A and B). This was also seen at lower concentrations of 1400W (0.1 μ M), although not significantly (asthma: 1.52 [4.03-1.34] vs. controls 1.19 [0.57-1.66], DPM/ μ g protein; $p=0.11$; (data not shown). There were no significant effects of 1400W on the synthesis of the other proteoglycans; perlecan ($p=0.13$), biglycan ($p=0.12$) and decorin ($p=0.12$) in distal fibroblasts from patients with asthma compared to control subjects (fig 3C-

H). The unselective NOS-inhibitor L-NAME and the NO donor DETA-NONOate did not show any significant effects on proteoglycan production from either fibroblasts from controls or patients with asthma (Fig 3A-H).

4. DISCUSSION

In our present work we investigated if NO affects synthesis of the specific ECM proteins, proteoglycans. The selective iNOS inhibitor 1400W significantly increased versican production in distal lung fibroblasts from patients with mild asthma. Interestingly, no increase of any proteoglycans was seen in the distal lung fibroblasts from healthy subjects. This data supports the notion of an altered, more matrix producing fibroblast phenotype in asthma. In our previous study patients with mild asthma presented higher alveolar NO in their exhaled air compared to the healthy controls (2). In this study we could show a tendency to higher FeNO₅₀ values in the mild asthmatic group compared to the healthy subjects. Asthmatic patients, uncontrolled on standard ICS therapy, have been shown to have increased alveolar NO levels (19, 28, 29). Increased alveolar NO may indicate a greater risk for asthma deterioration with nocturnal awakening (30) and risk for exacerbations and increased annual decline in lung function (31, 32). It is thus plausible to assume that nitric oxide in the peripheral airways is involved in the process of airway inflammation and remodelling, associated with different mechanisms compared to more central airways. Interestingly, distal lung fibroblasts are one source of synthesized alveolar NO and these cells express the iNOS enzyme as shown by mRNA data in the present study. Our iNOS mRNA data revealed that the fibroblasts obtained from asthmatic subjects had a tendency to increased iNOS gene activation compared to the control subjects, and specific iNOS inhibition significantly reduced NO synthesis in the distal fibroblasts from the asthmatic subjects. Higher iNOS production has been seen in another study with central lung fibroblasts from patients with

asthma, although with a small number of individuals (33). Our obtained data could implicate that the ongoing inflammation and remodelling processes, as we previously seen in the distal lung (2), may cause the induction of iNOS in these asthmatic subjects. In our present study the selective iNOS inhibitor 1400W significantly increased proteoglycan synthesis in distal fibroblasts from asthmatic subjects. The unselective NOS inhibitor L-NAME did not reach the same potential inhibition on proteoglycan production as the selective iNOS inhibitor. Interestingly, L-NAME has been shown to be specific for constitutively expressed NOS (eNOS and nNOS) at lower dose range 4-65 μ M whereas higher doses of L-NAME is required for iNOS inhibition (34), implicating that iNOS is the main enzyme responsible for the effects seen in the present study. However, our pilot experiments with higher doses of L-NAME (100 μ M) did not differ from 30 μ M L-NAME (*data not shown*) and L-NAME has been shown to have weak effects also in patients studies with asthmatic subjects; L-NAME did not alter ventilation-perfusion in asthma (35), nor did it affect allergen-induced early and late asthmatic responses (36), whereas another unselective NOS inhibitor, when given by inhalation, caused a fall in exhaled NO in normal and asthmatic subjects (37) and protected against the bronchocontractile effects of bradykinin (38). Taken the data together, L-NAME does not seem to be the ideal unselective NOS inhibitor, although it is well known and used in numerous different experimental settings.

In the present study, exogenous NO did not significantly affect proteoglycan synthesis in lung fibroblasts from either asthmatic or control subjects, although there were some tendencies to lower proteoglycan synthesis in fibroblasts from patients with asthma. In other studies, higher doses of exogenous NO have been shown to affect proliferation rate (39) and collagen synthesis (40) and inhibit fibroblast-mediated gel contraction (41). However, high doses of NO may cause peroxynitrite formation, induce oxidative DNA damages and apoptosis (39) and impair collagen synthesis due to toxicity (40). Importantly, these high levels of NO

(>1mM) do not appear to mimic the low concentrations of NO produced in the distal lung as measured by the alveolar NO fraction in the exhaled air from the asthmatic patients in our study.

The increase of versican after iNOS inhibition may have several possible effects on the remodelling process. Versican has been found to be destructive for the elasticity in the lung by inhibiting elastin-binding protein thus interfering with the assembly of elastic fibres (42). Accumulation of versican could lead to increased stiffness in the lung thereby affecting lung function (43). Biglycan is sequestered in ECM under normal physiological conditions, and increase during tissue stress or injury thus inducing a wide range of effects. Biglycan and decorin can induce morphological and cytoskeletal changes in fibroblasts giving rise to a more migratory fibroblast phenotype, which may promote wound-healing processes (44). Proteoglycan syntheses are closely regulated by different matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP). Different studies show that iNOS inhibition may decrease MMP-2 and 9 protein levels and activation (33, 45) by down-regulating TIMP-2 activation (14), which could partly explain the effect of iNOS inhibition on proteoglycan synthesis in our present study. However, due to limited amount of material we were not able to measure any MMPs or TIMPs which otherwise could have further strengthen this hypothesis. Another possible explanation for the effect of iNOS inhibition could be that excess amounts of NO are bound to ECM proteins via s-nitrosylation of thiols (46) and NO has an affinity for proteoglycans due to cysteine residues in the core protein. In our previous study with more patients, the synthesis of proteoglycans perlecan, biglycan and decorin from distal lung fibroblasts all correlated negatively to alveolar NO in asthmatic subjects but no correlations were detected in healthy subjects. Interestingly, there was no correlation between alveolar NO and proteoglycan production in healthy controls (2). There may be possibility of other biochemical pathways potentially modulated by iNOS. The substrate for NO, L-

arginine, is catalysed in competition by both arginase and NOS, and their products have opposing biological effects. L-arginine metabolized through arginase yields ornithine, a precursor of proline and polyamines involved in cell growth and proliferation (47). As glucocorticoids have been shown to downregulate iNOS, arginase is instead upregulated (48) and arginase activity has been shown to be upregulated in asthmatic subjects (49). Maarsingh et al showed that arginase is involved in allergen-induced airway remodelling, inflammation and hyperresponsiveness in an animal model of chronic asthma (50). It is tempting to speculate that arginase could be responsible for the enhanced synthesis of proteoglycans after iNOS inhibition in fibroblasts from asthmatic subjects, although it has to be proven in future studies.

In the present study, distal control lung fibroblasts were obtained from healthy subjects of mixed age and different sampling techniques due to limited access to transbronchial biopsies from healthy individuals. The donor lungs from the healthy individuals had been judged by the clinicians to be suitable for lung transplantation and we received the lungs due to the fact that they could not find any matching recipients at the moment. Despite the limited numbers of observations in the present study, we could support the findings in our other studies (21, 22) that the different sampling techniques and the age distribution in the two study control populations did not interfere with the obtained results. However, it would be plausible to have lung function data and exhaled NO levels from all the controls, i.e. also the explanted lungs. In our present study fibroblasts were derived from patients with mild asthma that did not take any ICS therapy. Asthmatic airways are characterized by airway remodelling, and these anatomical changes have been shown to play a mechanistic role in airway narrowing (2, 15, 16). It may therefore appear counterintuitive that fibroblasts from patients with asthma, with higher iNOS mRNA and NO (Fig 2), demonstrated an increased proteoglycan synthesis when iNOS was inhibited. Clinically, glucocorticosteroids are indirect inhibitors of iNOS synthesis

and exhaled NO in asthma (11, 13). Importantly, in our previous study where we investigated the effect of ICS (budesonide) on ECM production in bronchial fibroblasts from patients with asthma we could show that budesonide had almost none effect on ECM synthesis (51). We could also demonstrate that lung tissue composition differs between patients with controlled and uncontrolled asthma on equivalent doses of ICS, which support the impression that patients who have remaining symptoms despite conventional ICS therapy have a pronounced ECM remodelling in both central and distal airways (52). In another study with the glucocorticoidsteroid dexamethasone, ECM was decreased in fibroblasts from healthy control subjects but not from patients with asthma that was related to increased expression of activator protein-1 (AP-1) transcription factor in fibroblasts from patients with asthma. Excessive amounts of AP-1 may decrease the number of activated GR in nuclei and thereby impair ICS effects on gene transcription (53). Taken all the data together, this would suggest that chronic steroid treatment could lead to proteoglycan accumulation into the airways and promote airway remodelling. It is therefore tempting to speculate that NO could have a modulatory role in distal airway remodelling. In support of a modulatory effect of NO in distal airways, iNOS inhibition was found to cause enhanced antigen-induced contractions and increased cysteinyl-leukotriene release in guinea pig lung parenchymal strips (54), whereas addition of exogenous NO showed modest dilatory effect in the distal lung compared to more proximal airways and pulmonary arteries (55, 56). In a transgenic mice model overexpressing iNOS, NO was found to have beneficial effects on lung function and that endogenous NO *per se* did not cause airway inflammation (57). However, in an acute vs. chronic *in vivo* mouse model of ovalbumin-induced airway inflammation different outcomes were seen (14), implicating that iNOS have different roles during the acute and chronic inflammation. NO appears to be a double-edged sword in the physiology and pathology of the

human airways and many important questions regarding these messengers and signalling molecules remain to be answered.

5. CONCLUSIONS

In conclusion, specific iNOS inhibition significantly reduced NO synthesis and increased synthesis of the proteoglycan versican in distal lung fibroblasts from asthmatic subjects compared to fibroblasts from control subjects. These data suggest that NO could have a modulatory role in the pathological matrix remodelling that occurs in the distal lung in asthmatic subjects. The link between iNOS and distal airway remodelling could have clinical implications and further supports the notion of small airways as a desirable target for asthma management.

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FIGURE CAPTIONS

Figure 1. Measurements of exhaled NO. Measurements of exhaled NO: FeNO₅₀ (A) bronchial flux of NO (B) and alveolar NO (C) in patients with asthma (n=6) and healthy controls (n=5). The figure presents individual scores and median.

Figure 2. Expression of iNOS mRNA and NO synthesis in distal lung fibroblasts. Expression of iNOS mRNA in distal lung fibroblasts from patients with mild asthma (n=6) and healthy control subjects (n=8, 2A). iNOS mRNA data are presented as $2^{\Delta C_t} \times 10^5$ determined by subtracting the C_t values for iNOS from the C_t value for the housekeeping gene ($=\Delta C_t$). NO synthesis in distal lung fibroblasts from patients with mild asthma (n=6) and healthy control subjects (n=8) measured indirectly by chemiluminescence as nitrite/nitrate (B). Nitrite/nitrate levels produced from fibroblasts from patients with mild asthma (C) and healthy control subjects (D) after treatment with the selective iNOS inhibitor 1400W 1 μ M, the unselective NOS inhibitor L-NAME 30 μ M or the NO donor DETA-NONOate 10 μ M. Data are presented as fold increase of nitrite/nitrate production over non-stimulated cells (0.4% serum) presented (B). *depicts $p < 0.05$. Data are presented as median values together with interquartile range.

Figure 3. Proteoglycan synthesis in distal lung fibroblasts. Distal lung fibroblasts from patients with mild asthma (n=6) and healthy control subjects (n=8) were treated with the selective iNOS inhibitor 1400W, the unselective NOS inhibitor L-NAME or the NO donor DETA-NONOate. Quantification of proteoglycans was done after measuring incorporation of ³⁵sulphate using a scintillation counter, separated by SDS-PAGE and quantified using densitometry and presented as DPM/μg protein. Individual proteoglycan production of versican (A, B), perlecan (C, D), biglycan (E, F) and decorin (G, H) was measured from distal fibroblasts from patients with asthma (A, C, E, G) and healthy subjects (B, D, F, H). Data are presented as median values together with interquartile range and presented as fold increase of proteoglycan production over non-stimulated cells (0.4% serum). * depicts p<0.05.

Figure 1

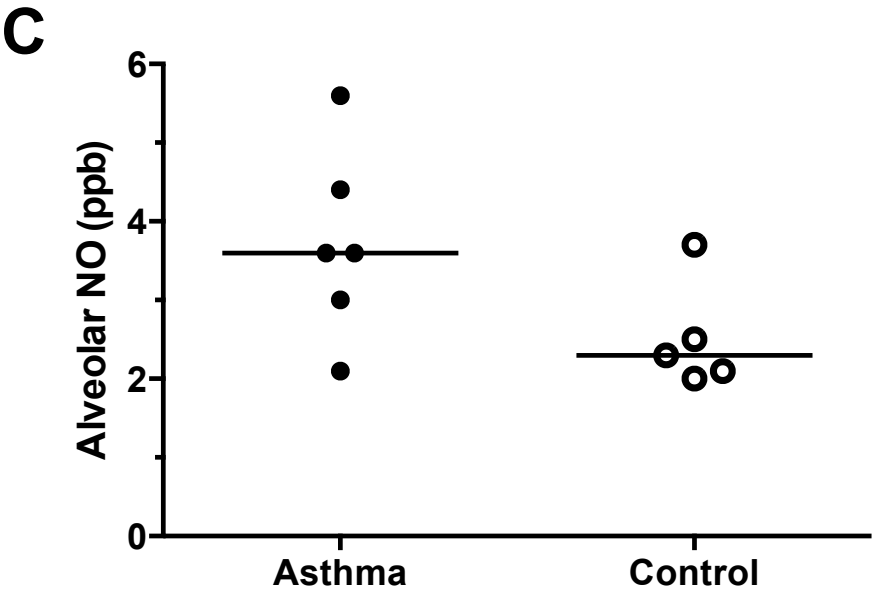
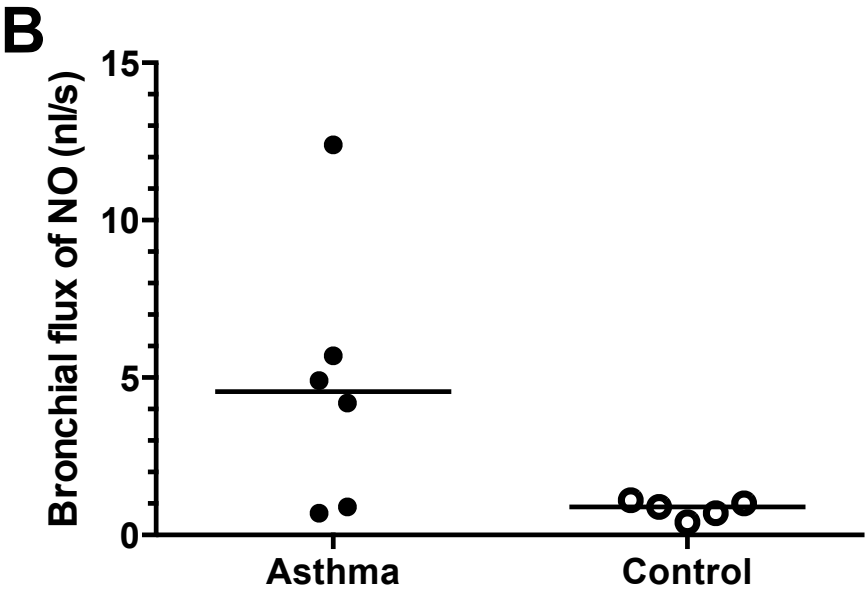
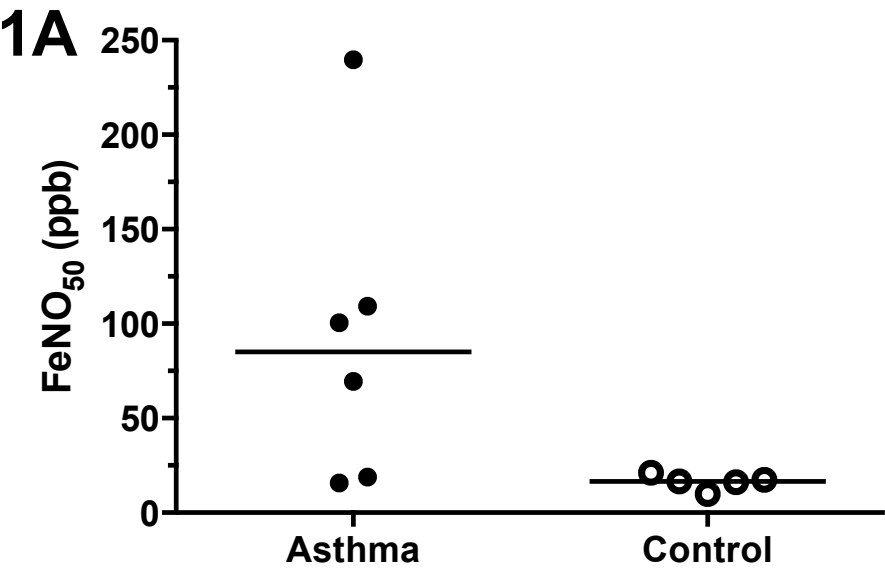


Figure 2

Fig 2

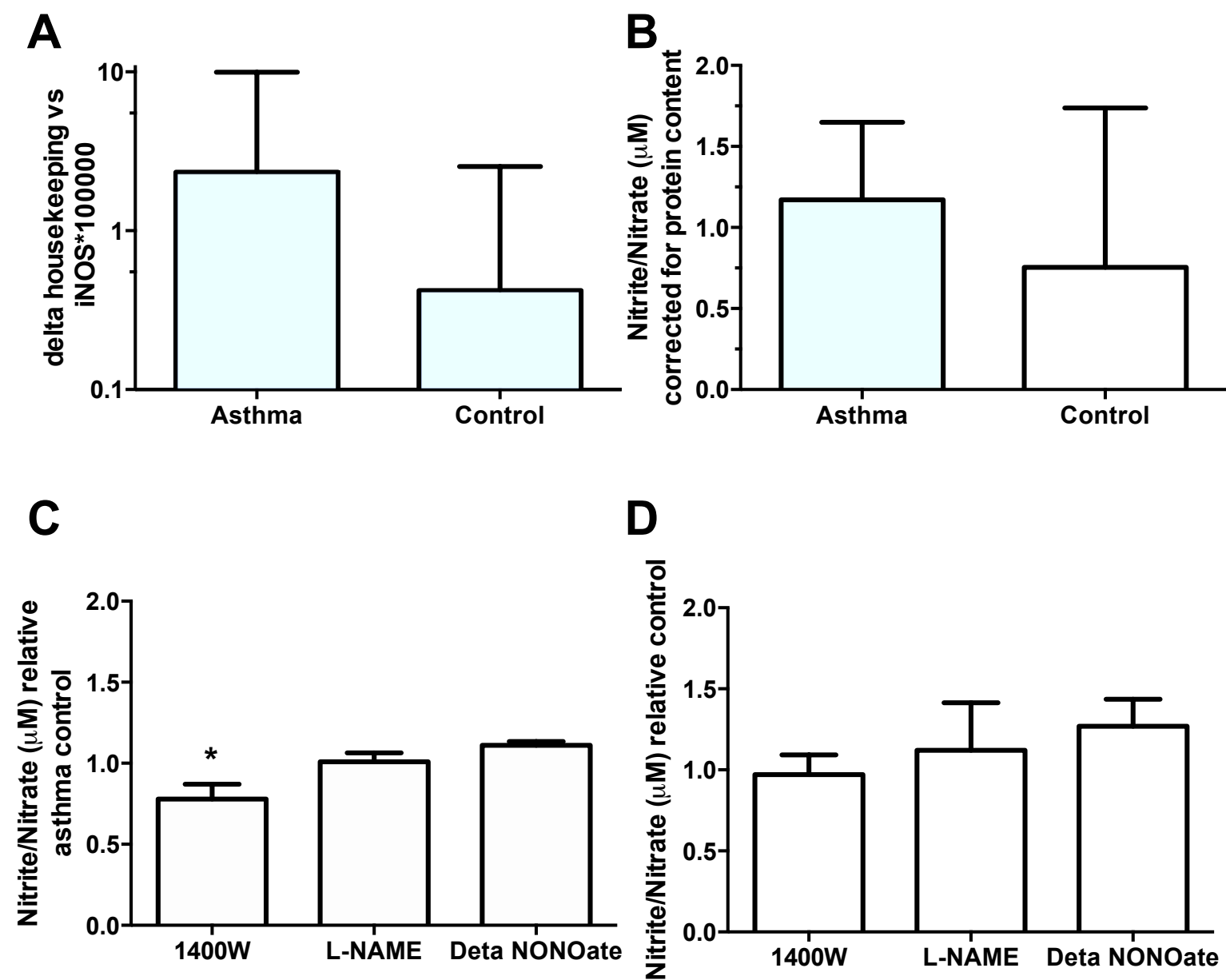
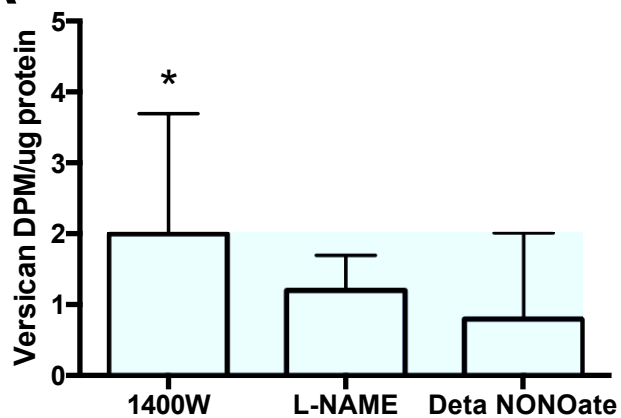
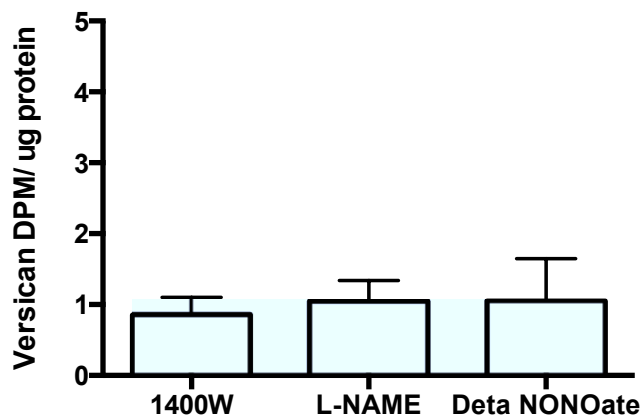


Figure 3

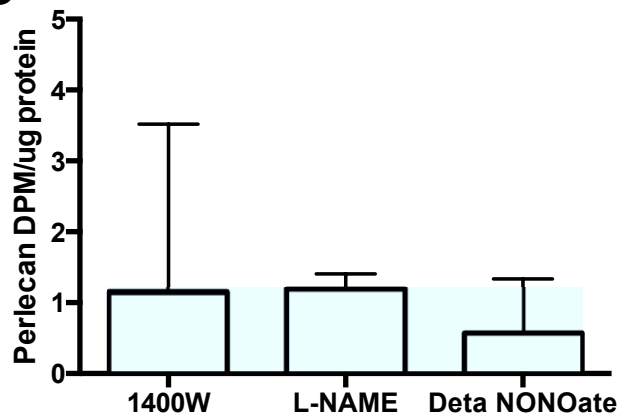
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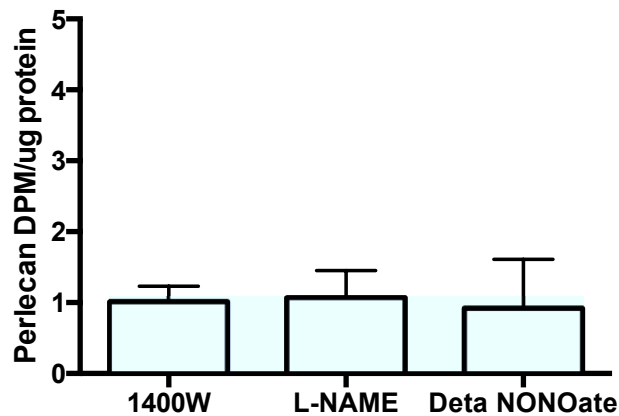
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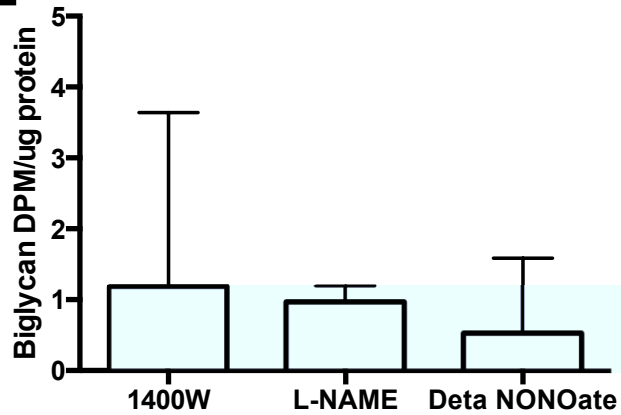
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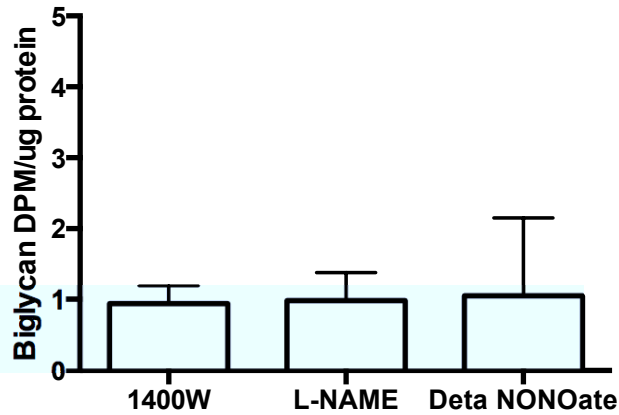
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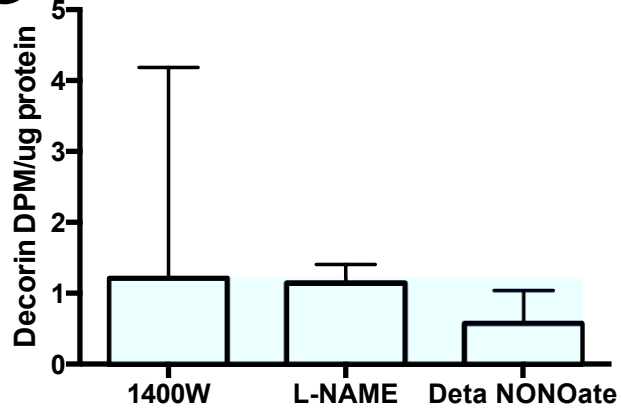
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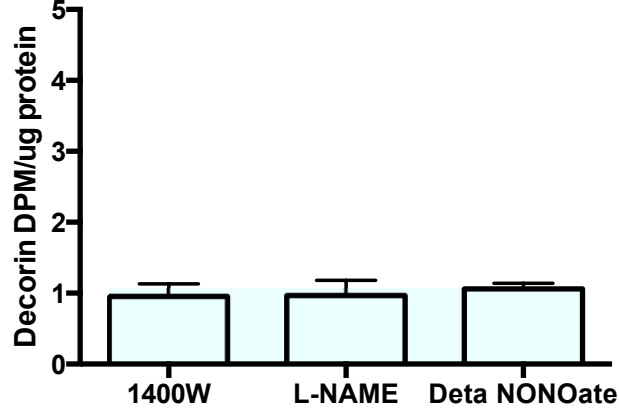
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H



Supplement: Fig 1

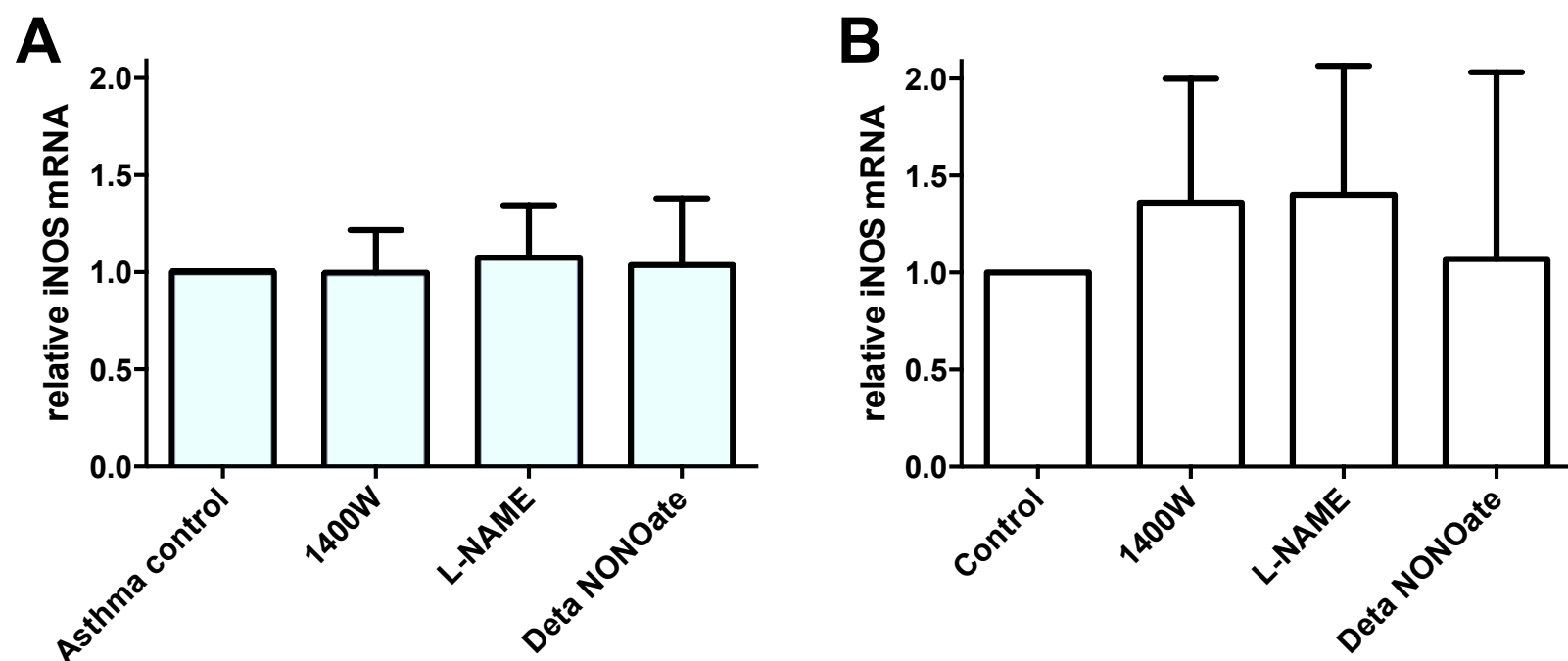


Figure 1. Expression of iNOS mRNA in distal lung fibroblasts. Expression of iNOS mRNA in distal lung fibroblasts from patients with mild asthma (n=6, A) and healthy control subjects (n=8, B) presented as fold change values after treatment with the selective iNOS inhibitor 1400W, the unselective NOS inhibitor L-NAME or the NO donor DETA-NONOate. Data are presented as $2^{\Delta C_t} \times 10^5$ determined by subtracting the C_t values for iNOS from the C_t value for the housekeeping gene ($=\Delta C_t$) as ratio of non-stimulated cells using Pfaffl method. Data are presented as median values together with interquartile range.