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Inhaled mannitol shifts exhaled nitric oxide in opposite directions in asthmatics and healthy subjects

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Abstract

We investigated if healthy subjects could release NO upon hyperosmolar challenge as a defence mechanism, and whether asthmatics with atopy showed an altered response. A plot of NO output versus flow rate was used to calculate the alveolar level and the NO-flux from the airways. The asthmatics had a higher NO output and this was due to an increased NO-flux from the airways, 86 ± 30 nl min^{-1} compared with control 21 ± 2 nl min^{-1} ($P < 0.05$). The alveolar NO levels showed no difference. In response to a dry powder of mannitol the exhaled NO concentration decreased in asthmatics by $37 \pm 7\%$, but increased in the control by $9 \pm 4\%$ ($P < 0.001$). The $FEV_{1.0}$ decreased $13 \pm 2\%$ and airway conductance $42 \pm 7\%$ in asthmatics and in the controls $2 \pm 1\%$ and $0 \pm 7\%$, respectively ($P < 0.001$). We conclude that asthmatics have an altered response to mannitol challenge in regards to exhaled NO. This may result from down regulation of constitutive NO production as a result of high levels of NO flux from the airways.

Keywords: Disease, asthma; Mammals, humans; Mediator, NO; Pharmacological agents, mannitol; Upper airways, inflammation

1. Introduction

Nitric oxide (NO) has many regulatory functions in the human body. It is known that inhaled NO can reduce the response to inhaled methacholine in both animals and humans (Högman et al., 1993a,b). In 1993 Persson and Gustafsson

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reported that NO was released upon airway provocation in a guinea pig model (Persson and Gustafsson, 1993) and appeared to act as a defence molecule. Blocking the NO production from the airway results in airway hyperresponsiveness in guinea pigs (Nijkamp et al., 1993). Hyperresponsiveness is a hallmark of asthma. Paradoxically, exhaled NO levels in asthmatics are not reduced, but markedly elevated compared to healthy controls (Alving et al., 1993). The exhaled NO levels are said to reflect the severity of the disease (Massaro et al., 1995) and can be reduced by treatment with inhaled glucocorticoids (Kharitonov et al., 1996a). These findings suggest that an inflammatory response in the airways is the cause for the increase in exhaled NO in asthma. Folkerts et al. (1995) showed in a guinea pig model infected with parainfluenza type 3 virus that the airways became hyperresponsive to histamine and upon challenge NO decreased. This was a different response to the healthy animals where an increase in the NO release occurred due to histamine. Because both the hyperresponsiveness and the decrease in NO release could be prevented by preincubation of the tissue with L-arginine, the authors concluded that the hyperresponsiveness was due to NO deficiency.

We hypothesised that subjects with airway hyperreactivity are not able to release NO in response to an airway insult. Thus, we investigated if healthy subjects released NO upon hyperosmolar challenge and whether asthmatic subjects showed an altered response. Dry powder inhalation of mannitol was chosen because it is an indirect challenge affecting the epithelial cells (Koskela et al., 2000) we wanted to study.

2. Methods

2.1. Subjects

All the subjects with allergic asthma regularly attended the Department of Respiratory Medicine and Allergology at the University Hospital for treatment of their asthma. All of them were non-smokers, atopic, had respiratory symptoms during the last 12 months, a forced expiratory volume in one second ($FEV_{1.0}$) above 70% of the predicted value, and had previously demonstrated an increased responsiveness to inhaled methacholine. The methacholine was given in ten successively increasing doses ranging from 0.0625 to 32 mg ml^{-1} delivered by an automatic, inhalation synchronised dosimeter jet nebuliser, Spira Elektro 2 (Respiratory Care Centre, Hameenlinna, Finland) (Nieminen et al., 1988). Patients with a provocative concentration of methacholine of ≤ 32 mg ml^{-1} and a decrease in $FEV_{1.0}$ of 20% or more (PC_{20}) were diagnosed as having a positive test. Atopy was defined as at least one positive skin prick test to common allergens. Skin prick tests were carried out using Soluprick® (ALK-Abelló A/S, Hørsholm, Denmark) standardised allergen extract against the following allergens: birch, timothy grass, mugwort, cat, dog, horse, *Dermatophagoides pteronyssinus*, *Cladosporium* and *Alternaria*. A positive test was a reaction with a mean of >3 mm and no dermatographism.

Ten healthy subjects served as controls. These subjects had no airway symptoms, a negative skin-prick test and a negative methacholine test. The Human Ethics Committee at the University of Uppsala approved the protocol. All the subjects were informed of the reasons for the study and written consent was obtained.

2.2. Protocol

The subjects attended the laboratory twice during a time period of less than two weeks. At the first visit exhaled NO levels at different expiratory flow rates were obtained then followed a lung function test and a provocation with inhaled dry powder mannitol. Exhaled NO at a flow rate of 0.25 L s^{-1} ($FENO_{0.25}$) and a forced expiration with measurement of $FEV_{1.0}$ and flow–volume loops were obtained after each dose of mannitol. A second lung function test followed immediately after the mannitol challenge.

At the second visit exhaled NO levels at different expiratory flow rates were obtained, a placebo challenge (using the same number of empty capsules as for the mannitol challenge) was performed together with measurement of $FEV_{1.0}$, flow–volume loops and $FENO_{0.25}$. Lung function

tests were done before and after the placebo challenge. Sputum was induced at the end of this visit.

2.3. Mannitol capsule challenge

The preparation of the dry powder mannitol has been described in detail previously (Anderson et al., 1997). In brief, mannitol (Mannitol BP, Rhône Poulenc Chemicals Pty. Ltd., Brookvale, NSW, Australia) capsules were hand-filled with 5, 10, 20 and 40 mg of mannitol powder as required under control conditions. The capsules were stored in a container with silica gel and kept in a cool dry environment. An inhalator (Boehringer Ingelheim Pharmaceuticals Inc., Providence, RI) was used for the delivery of the mannitol.

The challenge started with a baseline measurement of $FEV_{1.0}$ and this value was compared to the value obtained during the lung function test to confirm that it was stable. The asthmatic subjects then performed the challenge with doses consisting of 0 (empty capsule acting as a placebo), 5, 10, 20, 40, 80, 160 mg of mannitol via the dry powder inhaler. The 80 and 160 mg were given in multiple doses of 40 mg capsules. $FENO_{0.25}$ was obtained at the first breath after the inhalation of mannitol. At least two $FEV_{1.0}$ manoeuvres were performed 60 sec after each dose and the highest $FEV_{1.0}$ was used in the calculation. The $FEV_{1.0}$ value measured after the 0 mg capsule was taken as the prechallenge $FEV_{1.0}$ and used to calculate the percentage decrease in $FEV_{1.0}$ in response to the mannitol challenge. The asthmatic subjects were allowed to have 10% fall in $FEV_{1.0}$ in response to a single dose but never more than a 15% fall in $FEV_{1.0}$. For safety the oxygen saturation by pulse oximetry (SpO_2) and the heart rate were monitored during the challenge (CS/3, Datex-Ohmeda, Helsinki, Finland). On completion of the challenge, asthmatics received 0.5 mg terbulatine sulfate actuated and inhaled from a Nebunette (Draco, Lund, Sweden) and rested 30 min after the challenge and had to have a $FEV_{1.0}$ that was within 5% of their baseline value before leaving the clinic.

The healthy subjects performed an identical challenge, but here the cut off point was not a decrease in $FEV_{1.0}$ but a cumulative dose of mannitol of 155 mg.

2.4. Lung function measurements

The subjects performed the lung function measurements with a Masterlab Trans spirometer and a Masterlab body plethysmograph (Eric Jaeger AG, Würzberg, Germany). $FEV_{1.0}$, airway conductance (sGaw) and the slope of the curve for the fall in $FEV_{1.0}$ during the mannitol challenge were calculated.

2.5. NO analysing equipment and measurements

NO, flow rate and airway pressure were measured with a computer-based single-breath NO system from Nitrograf AB, Hässelby, Sweden. Included in this system is a Sievers NOA 280 chemiluminescence analyser (Sievers, Boulder, CO). The sampling rate of the NO system was adjusted to 200 ml min^{-1}. The repeatability of the system was < 1 ppb with a response time of < 200 ms. The system was calibrated using a mixture of 530 ppb NO in nitrogen (AGA AB, Lidingö, Sweden). The zero was set by feeding synthetic air (AGA AB) into a 2 L canister filled with Purafil II chemisorbant with purakol (Lindair AB, Ljusne, Sweden). Care was taken that the sampling flow of the analysing probe was kept the same as the flow at which the system was calibrated. A flow indicator was therefore attached to the system and sampling flow was controlled before and after each measurement. The temperature of the photomultiplier tube of the analyser differed less than 1°C from the temperature at which the calibration was performed. Because the NO signal is expiratory flow dependent (Högman et al., 1997; Silkoff et al., 1997) the NO system also includes measurements of expiratory flow rate. The flow sensor, D-lite™ (Datex-Ohmeda, Helsinki, Finland) was calibrated in the range of 0.025–0.500 L sec^{-1} (Dry Cal DC-2 flow calibrator, BIOS International, Pompton Plains, NJ). Controls of calibration and flow rate of the sampling system were done daily and the zero was controlled before each measurement.

The subjects were instructed to perform a deep inhalation and exhale with a constant flow. Five different expiratory flows between 0.05 and 0.32 L min^{-1} were used. To facilitate for the volunteers

to keep a constant expiratory flow, a resistance (model no. 7100R 20 or model no. 7100R 50, Hans Rudolph, Inc., Kansas City, MO) was fitted onto the expiratory side of the 2-way, non-rebreathing valve (model 1410, Hans Rudolph). The expiratory pressure was 5 cmH_2O or above but always < 20 cmH_2O to exclude NO from the nasal cavity (Kharitonov et al., 1996b; Silkoff et al., 1997). The expiratory pressure has been shown not to influence the NO concentrations (Högman et al., 1997; Silkoff et al., 1997). A mean value of three breaths was used for statistical analysis. The concentration of NO is expressed as FENO in ppb and the volume of exhaled NO is expressed as $\dot{V}$ENO in nl sec^{-1}. $\dot{V}$ENO was calculated by multiplying the FENO plateau values by the expiratory flow rate ($\dot{Q}$, L sec^{-1}).

We applied a model by Tsoukias and George (1998) to divide the exhaled NO into alveolar contribution (F_ANO, ppb) and airway flux of NO (nl sec^{-1}) in order to characterise our subjects. For each subject linear least squares was used to determine the best fit line through a plot of $\dot{V}$ENO versus $\dot{Q}$ to determine an estimate of F_ANO and the NO-flux from the airways. See Fig. 1 for an example.

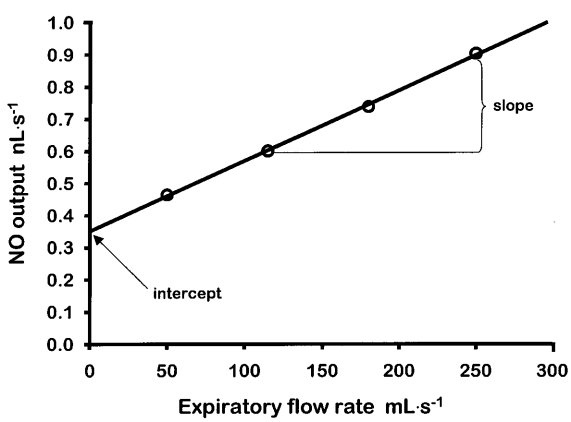

Fig. 1. An example of how to model alveolar NO contribution (slope) and the NO-flux from the airways (intercept) from a plot of $\dot{V}$ENO vs. $\dot{Q}$ after Tsoukias and George (1998).

2.6. Sputum induction and analysis

Sputum analyses were performed in order to characterise our study subjects. The subjects inhaled 3% solution of sodium chloride via an Omron U1 (Omron Healthcare GmbH, Hamburg, Germany) over a 15-min period. SpO_2 was monitored during inhalation of the saline aerosol and the patients were closely attended to avoid undue bronchoconstriction by saline aerosol.

The weight of the sputum sample was recorded and dithiothreitol 0.1% (Sputolysin, Calbiochem, CA) was added in a volume equal to the weight of the sputum sample. The sample was mixed for 15 min at room temperature, and then centrifuged for 10 min at $600 \times g$. The cell pellet was mixed with 10 ml PBS with citrate (0.01 mol l^{-1}), and then centrifuged for 10 min at $200 \times g$. The supernatant was removed, the cells were suspended in PBS with citrate and 2% (v/v) new borne calf serum (NBS), and counted and the viability was evaluated by the use of trypan blue. Cytospin slides were prepared for immunohistochemistry and differential count. The slides for differential count were stained with May-Grünewald and Giemsa solutions. The slides for immunohistochemistry were fixed in acetone for 3 min, left to dry for one hour, and then stored at -70°C for later analysis.

2.7. Statistical analysis

T-test for paired samples and ANOVA for repeated measurements were used to compare data within the groups. The Tukey honest significant difference test was used for post hoc comparisons and probability values were calculated. Between groups the Mann–Whitney *U*-test was applied. For correlation the Spearman rank correlation coefficient was used. For all statistical calculations the Statistica/w 5.0 software package (StatSoft Inc., Tulsa, OK) was used. A *P*-value of < 0.05 was regarded as statistically significant. Results are given as mean values $\pm$ S.E.M. in text, tables and Fig. 2.

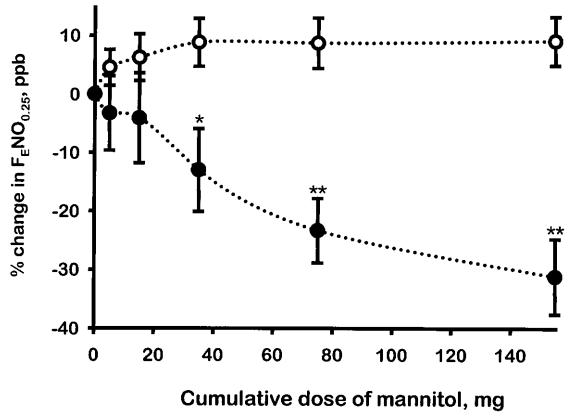

Fig. 2. The percentage changes in exhaled NO at an expiratory flow rate of 0.25 L min^{-1} ($FENO_{0.25}$) during mannitol provocation are shown for asthmatics (●) and control subjects (○). The response to mannitol by the asthmatics is significantly different from the control subjects after the third dose of mannitol. * $P < 0.05$, ** $P < 0.001$.

3. Results

3.1. Subject characteristics

Six of the ten asthmatic subjects were taking inhaled corticosteroids on a daily basis. Individual values of PD_{20} to methacholine, dose of inhaled corticosteroids, airway conductance (sGaw), $FEV_{1.0}$, exhaled NO levels at an expiratory flow rate of 0.1 L sec^{-1} ($FENO_{0.1}$) and the fraction of cells in sputum are shown in Table 1. The corresponding values for the controls are also shown as a mean value in Table 1.

There was no significant difference in $FENO_{0.1}$ between the two visits (asthmatics 16.0 ± 4.3, 16.6 ± 5.5 ppb; controls 5.9 ± 0.5, respectively, 5.6 ± 0.5 ppb). Neither were there any differences between the first and the second visit with regards to $FEV_{1.0}$ (asthmatics 4.0 ± 0.3, respectively, 3.9 ± 0.4 L; controls 3.7 ± 0.3, respectively, 3.6 ± 0.3 L) or sGaw (asthmatics 1.3 ± 0.1, respectively, 1.3 ± 0.1 (kPa sec)$^{-1}$; controls 1.8 ± 0.2, respectively, 1.9 ± 0.2 (kPa sec)$^{-1}$). The fraction of cells in the sputum showed a clear difference between the asthmatics and the control subjects (Table 1).

There was a significant difference in the mean value for $FENO_{0.1}$ between asthmatics and controls ($P < 0.05$), see Table 1. The $FENO_{0.25}$ also showed a difference ($P < 0.05$) between asthmatics and controls (8.5 ± 2.5, 3.1 ± 0.2 ppb) respectively. Calculating the mean F_ANO between the visits showed no difference between asthmatics and control (2.3 ± 0.5, respectively, 1.8 ± 0.2 ppb). However, the NO flux from the airways was significant higher for the asthmatics, 86.1 ± 29.8, compared with the controls, 21.0 ± 1.8 nl min^{-1} ($P < 0.05$). The group of asthmatics treated with inhaled steroids had a NO-flux of 85 ± 44 and the untreated asthmatics had a NO flux of 88 ± 43 nl min^{-1} (ns).

There was a correlation between the eosinophil-fraction in the sputum and the NO flux from the airways for all the subjects studied, $r = 0.60$, $P < 0.01$. The correlation between neutrophil-fraction in the sputum and the NO flux was negative, $r = -0.56$, $P < 0.01$. There was no correlation between F_ANO and the fraction of the sputum cells.

3.2. Mannitol challenge

There was a clear difference between asthmatic and the control subjects in the reaction to the same dose of inhaled mannitol for the change in $FEV_{1.0}$, sGaw and exhaled NO values ($P < 0.001$). When calculated the slope of the curve for the percentage fall in $FEV_{1.0}$ to the dose of mannitol resulted in a slope of -0.05 ± 0.02 for asthmatics and 0.0 ± 0.01 for controls ($P < 0.05$). The maximal dose of inhaled mannitol ranged from 45 to 475 mg with a mean value of 300 ± 35 mg for the asthmatics. The asthmatics had a fall in $FEV_{1.0}$ from 4.0 ± 0.3 to 3.5 ± 0.3 L ($P < 0.001$). Challenge with placebo in the asthmatics caused a decrease of $FEV_{1.0}$ from 3.9 ± 0.4 to 3.8 ± 0.4 L ($P < 0.05$). The sGaw decreased due to mannitol in the group of asthmatics from 1.3 ± 0.1 to 0.7 ± 0.1 (kPa sec)$^{-1}$ ($P < 0.001$). There was also a decrease in sGaw during placebo challenge in the group of asthmatics, from 1.3 ± 0.1 to 1.1 ± 0.1 (kPa sec)$^{-1}$ ($P < 0.01$). The $FENO_{0.25}$ decreased significantly due to inhalation of mannitol for the asthmatics, from 8.0 ± 2.1 to 4.8 ± 1.2 ppb ($P < 0.05$). After placebo challenge the $FENO_{0.25}$ did not change significantly, from 7.3 ± 0.2 to 6.9 ± 0.2 ppb. The percentage change in $FENO_{0.25}$ for

Table 1
Characteristics of the asthmatic subjects and the control subjects participating in the study.

	Gender (F/M)	Age (years)	MCh PD_{20} (mg)	Cortisone (μg day^{-1})	sGaw $(kPa\ sec)^{-1}$	$FEV_{1.0}$ (% pred)	NO ppb (0.1 L sec^{-1})	Fraction of cells in sputum			
								Neutrophils	Eosinophils	Lymphocytes	Macrophages
Patient 1	F	23	0.3	400	0.8	95	10.0	49.3	11.1	11.0	24.7
Patient 2	M	29	1.7	0	1.3	105	12.5	30.0	0	8.3	13.7
Patient 3	F	32	0.1	800	1.3	102	40.3	23.9	10.0	6.6	25.1
Patient 4	M	28	2.5	0	1.5	100	9.3	46.3	6.8	8.1	31.6
Patient 5	F	38	0.5	400	1.8	100	6.0	59.5	1.5	4.2	30.3
Patient 6	F	21	0.1	0	1.4	79	14.0	16.2	1.7	6.7	65.3
Patient 7	M	20	1.9	0	1.7	132	42.6	32.6	10.9	6.9	40.4
Patient 8	F	37	0.5	800	1.3	86	6.5	39.3	4.7	7.0	39.7
Patient 9	F	24	5.0	400	0.8	86	7.6	–	–	–	–
Patient 10	F	36	0.6	400	1.1	98	11.6	17.2	11.4	4.6	77.0
Mean ± S.E.M.	7F/3M	29 ± 2	1.3 ± 0.5	–	1.3 ± 0.1	98 ± 5	16.0 ± 4.3	35 ± 5	6 ± 2	7 ± 1	39 ± 7
Subjects											
Mean ± S.E.M.	8F/2M	41 ± 3	neg	–	1.8 ± 0.2	102 ± 4	5.9 ± 0.5	77 ± 5	1 ± 1	3 ± 1	11 ± 4
Statistics: asthmatics vs. controls					$P<0.05$	ns	$P<0.01$	$P<0.001$	$P<0.01$	$P<0.01$	$P<0.01$

all subjects during mannitol and placebo challenge can be seen in Table 2 and in Fig. 2.

The control subjects inhaled 155 mg of mannitol. There was no change in the $FEV_{1.0}$ in the control group due to mannitol challenge, 3.7 ± 0.3, respectively, 3.6 ± 0.3 L. Neither was there a change in $FEV_{1.0}$ due to the placebo challenge, 3.6 ± 0.3 L. No change of the sGaw values was observed for the control group after mannitol or placebo challenge, 1.8 ± 0.2 (kPa sec)$^{-1}$. A small increase was seen in the $FENO_{0.25}$ due to the mannitol challenge in the control subjects, from 3.1 ± 0.3 to 3.5 ± 0.4 ppb ($P < 0.05$). After placebo challenge the $FENO_{0.25}$ did not significantly change in the control group from 2.9 ± 0.3 to 3.0 ± 0.3 ppb.

In the present study we noted that the asthmatics had a reduction in FENO. This reduction was not related to how long (time) the patient had had their diagnosis of asthma, whether they were taking inhaled steroids or their pre-challenge level of lung function. The reduction in $FENO_{0.25}$ did not correlate to the decrease in sGaw. Individual analysis of a correlation between $FEV_{1.0}$ and NO during mannitol challenge showed that a rapid decrease in NO could be followed by a moderate decline in $FEV_{1.0}$.

4. Discussion

This study shows that during airway provocation with dry powder mannitol the asthmatics responded with a decrease in exhaled NO. This was clearly different to the response observed in the healthy subjects in whom the NO levels statistically increased in response to challenge. The exhaled NO expressed both as fraction of exhaled gas and as a flux from the airways was higher in the asthmatics compared with controls. The calculations of NO-flux suggest that the source of increased NO in asthmatics is the conducting airways and not the alveolar region. The finding that the asthmatics have a significant elevated fraction of inflammatory cells in the sputum is in keeping with the inflamed airways being associated with a higher flux of NO from the airways.

This is the first study to demonstrate that healthy subjects increase their exhaled levels of NO during an osmotic airway provocation. The asthmatic subjects decreased their NO levels in response to the same dose of dry powder mannitol. The finding of a decreased NO level in response to inhaling a hyperosmolar provocation such as mannitol confirms an earlier report showing that there is also a reduction in NO levels following provocation with hypertonic saline (de Gouw et al., 1998).

While a decrease in exhaled NO occurs in asthmatics with indirect stimuli such as adenosine and hypertonic saline (de Gouw et al., 1998) and now mannitol, there is conflicting data with regards to exhaled NO and direct stimulation of the bronchial smooth muscle by pharmacological agents. Histamine and methacholine have both been shown not to change NO levels (de Gouw et al., 1998; Kharitonov et al., 1998), although there is one study reporting a decrease in response to

Table 2
The response to mannitol inhalation by asthmatics and control subjects[a]

	sGaw% change		$FEV_{1.0}$% change		$FENO_{0.25}$% change	
	Mannitol	Placebo	Mannitol	Placebo	Mannitol	Placebo
Asthmatics	-42.0 ± 7.1*	-13.2 ± 3.2	-12.8 ± 2.4**	-3.4 ± 1.2	-36.8 ± 7.3*	-5.1 ± 3.6
Controls	-0.4 ± 6.8	-5.3 ± 4.9	-1.6 ± 0.7	-0.3 ± 1.3	9.2 ± 4.1*	0.4 ± 0.5

[a] The inhalation of mannitol was stopped in the group of asthmatics when the reduction in $FEV_{1.0}$ was more than 10%. The maximal dose of mannitol ranged from 45 to 475 mg in the asthmatics. The control subjects stopped the mannitol inhalation at a cumulative dose of 155 mg. Data are given in mean ± S.E.M.

* Mannitol vs. placebo: $P < 0.01$.

** Mannitol vs. placebo: $P < 0.001$.

methacholine (Henriksen et al., 1999). That airway narrowing can be induced without a change in exhaled NO suggests that changing the surface area of the airway mucosa does not necessarily affect the exhaled NO levels. Rather the functions of the airway epithelium will rapidly change the exhaled NO levels. In a hyperreactive upper airway mannitol elicits an epithelial cell response (Koskela et al., 2000) which may cause this change in NO levels. Many cellular and chemical constituents can be released when the airway is exposed to a hyperosmolar stimulus. These include histamine (Eggleston et al., 1990), neuropeptides (Högman et al., 1999) and arachidonic acid metabolites both through the 5-lipooxygenase (Silber et al., 1988) and the cyclooxygenase pathways (Gravelyn et al., 1988). Inhalation of PGE_2 and $PGF_{2\alpha}$ has been shown to decrease the exhaled NO (Kharitonov et al., 1998). The presence of substances, released into the airway as a result of a hyperosmolar stimulus, could lead to deactivation or simply scavenging of the NO molecule both at the epithelial level and/or by increased blood supply, and thereby explain the decrease in NO levels. By contrast, the increase in NO levels in the healthy subjects could simply be due to the effect of mannitol as a classical hydroxyl radical scavenging agent. Interestingly, it might also be that mannitol inhibits the breakdown of NO into peroxynitrous acid (Poullis, 1999). Further studies have to address these findings but should preferably be done with a lower expiratory flow rate or by NO modelling of the distribution of the contribution of NO from the alveolar region and airways. Using a high flow rate as in this study, only small changes in the exhaled NO were seen, because of the short time allowed for diffusion of NO from the airways. However, the European Respiratory Society Task Force 'measurement of nitric oxide in exhaled air' recommended this high flow rate at the time for the study.

It is intriguing that asthmatics have a higher flux of NO compared with controls yet be less able to induce release at a time the airways are provoked. One explanation for this could be that the asthmatics were not able to defend their airway upon challenge because of an incomplete or down-regulation of constitutive NO production due to high levels of exhaled NO. This concept would be in keeping with results found in animals. Thus when NO production was induced and high levels of inducible NO synthase mRNA was found in the tissue, there was a down-regulation of the endothelial NO synthase mRNA (Liu et al., 1996; Scott et al., 1996). Further down-regulation of constitutive NO production could result in increased airway responsiveness. Support for this theory is that asthmatics with high exhaled NO levels are more likely to react to airway provocation with indirect stimuli (Ludviksdottir et al., 1999). Inhaled glucocorticoids are known to decrease the levels of exhaled NO in asthmatics (Kharitonov et al., 1996c) and decrease the response to hyperosmolar stimuli (Rodwell et al., 1992). One possible explanation, among others, could be that the decrease in hyperresponsiveness seen after corticosteroid treatment (Molema et al., 1989) is due to an up-regulation of the constitutive NOS activity.

Again this concept is supported by findings in virus-infected guinea pigs, where airway hyperresponsiveness but no release of NO after provocation was demonstrated (Folkerts et al., 1995). Virus infected guinea pigs responded normally after they were given L-arginine, the substrate for NO synthase. The question arising from this is whether addition of NO to the inhaled air can improve the airway conductance in asthmatics. Indeed, this has been shown to be true (Högman et al., 1993b). In addition, both Högman et al. (1993b), Sanna et al. (1994) have shown that NO inhalation reduces the airway responsiveness in subjects with airway hyperresponsiveness.

Further, subjects who inhale large amounts of NO, i.e. smokers, have low levels of exhaled NO (Persson et al., 1994) possibly caused by a down-regulation of NO production or a rapid breakdown of the NO formed. Cigarette smoking is associated with an increased risk of airway hyperresponsiveness, respiratory tract infections and chronic airway disease, which points toward a compromised defence system.

In this study we have also shown that the NO-flux from the airways was increased in asthmatics by using a plot of $\dot{V}$ENO versus expiratory

flow rate. This type of analysis may be useful to determine the relative contribution of exhaled nitric oxide in many other inflammatory diseases of the lungs such as cystic fibrosis, Sjögren's syndrome, sarcoidosis, and idiopathic pulmonary fibrosis. All of these diseases have altered levels of exhaled nitric oxide, but we do not yet know the anatomical source for the exhaled NO.

We conclude that the reduction in NO levels observed in asthmatic, but not healthy subjects, in response to inhaled mannitol may result from down regulation of constitutive NO production as a result of high levels of NO flux from the airways. Such down regulation of an acute protective effect of NO could account for the airway narrowing resulting from provoking stimuli. Further we conclude that valuable information can be gained by measuring the anatomical source of NO in other inflammatory diseases of the lung.

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References

Alving, K., Weitzberg, E., Lundberg, J.M., 1993. Increased amount of nitric oxide in exhaled air of asthmatics. Eur. Respir. J. 6, 1368–1370.

Anderson, S.D., Brannan, J., Spring, J., Spalding, N., Rodwell, L.T., Chan, K., Gonda, I., Walsh, A., Clark, A., 1997. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. Am. J. Respir. Crit. Care Med. 156, 758–765.

de Gouw, H.W., Hendriks, J., Woltman, A.M., Twiss, I.M., Sterk, P.J., 1998. Exhaled nitric oxide (NO) is reduced shortly after bronchoconstriction to direct and indirect stimuli in asthma. Am. J. Respir. Crit.Care Med. 158, 315–319.

Eggleston, P.A., Kagey-Sobotka, A., Proud, D., Adkinson, N.F., Lichtenstein, L.M., 1990. Disassociatio of the release of histamine and arachidonic acid metabolites from osmotically activated basophils and human lung mast cells. Am. Rev. Respir. Dis. 141, 960–964.

Folkerts, G., van der Linde, H.J., Nijkamp, F.P., 1995. Virus-induced airway hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. J. Clin. Invest. 95, 26–30.

Gravelyn, T.R., Pan, P.M., Eschenbacher, W.L., 1988. Mediator release in an airway segment in subjects with asthma. Am. Rev. Respir. Dis. 137, 641–646.

Henriksen, A.H., Sue-Chu, M., Lingaas Holmen, T., Langhammar, A., Bjermer, L., 1999. Exhaled and nasal NO levels in allergic rhinitis: relation to sensitization, pollen season and bronchial hyperresponsiveness. Eur. Respir. J. 13, 301–306.

Högman, M., Frostell, C.G., Arnberg, H., Hedenstierna, G., 1993a. Inhalation of nitric oxide modulates methacholin-induced bronchoconstriction in the rabbit. Eur. Respir. J. 6, 177–180.

Högman, M., Frostell, C.G., Hedenström, H., Hedenstierna, G., 1993b. Inhalation of nitric oxide modulates adult human bronchial tone. Am. Rev. Respir. Dis. 148, 1474–1478.

Högman, M., Strömberg, S., Schedin, U., Frostell, C., Hedenstierna, G., Gustafsson, L.E., 1997. Nitric oxide from the human respiratory tract efficiently quantified by standardized single breath measurements. Acta Physiol. Scand. 159, 345–346.

Högman, M., Hageman, C., Hua, X.-Y., 1999. Hyperosmolar saline induces airway resistance changes and neuropeptide release: a comparison with the effect of capsaicin, potassium and histamine. Eur. J. Clin. Invest. 29, 264–269.

Kharitonov, S.A., Yates, D.H., Chung, K.F., Barnes, P.J., 1996a. Changes in the dose of inhaled steriod affect exhaled nitric oxide levels in asthmatic patients. Eur. Respir. J. 9, 196–201.

Kharitonov, S.A., Chung, K.F., Evans, D., O'Conner, B.J., Barnes, P.J., 1996b. Increased exhaled nitric oxide in asthma is mainly derived from the lower respiratory tract. Am. J. Respir. Crit. Care Med. 153, 1773–1780.

Kharitonov, S.A., Yates, D.H., Barnes, P.J., 1996c. Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. Am. J. Respir. Crit. Care Med. 153, 454–457.

Kharitonov, S.A., Sapienza, M.A., Barnes, P.J., Chung, K.F., 1998. Prostaglandins E_2 and $F_{2\alpha}$ reduce exhaled nitric oxide in normal and asthmatic subjects irrespective of airway caliber changes. Am. J. Respir. Crit. Care Med. 158, 1374–1378.

Koskela, H., Di Sciascio, M.B., Anderson, S.D., Andersson, M., Chan, H.-K., Gadalla, S., Katelaris C., 2000. Nasal hyperosmolar challenge with a dry powder of mannitol in

patients with allergic rhinitis. Evidence for epithelial cell involvement. Clin. Exp. Allergy 30 (11), 1628.

Liu, S.F., Adcock, I.M., Old, R.W., Barnes, P.J., Evans, T.W., 1996. Differential regulation of the constitutive and inducible nitric oxide synthase mRNA by lipopolysaccaride treatment in vivo in the rat. Crit. Care Med. 24, 1219–1225.

Ludviksdottir, D., Jansson, C., Högman, M., Hedenström, H., Björnsson, E., Boman, G., 1999. Exhaled NO and its relationship to airway responsiveness and atopy in asthma. Respir. Med. 93, 552–556.

Massaro, A.F., Gaston, B., Kita, D., Fanta, C., Stamler, J.S., Drazen, J.M., 1995. Expired nitric oxide levels during treatment of acute asthma. Am. J. Respir. Crit. Care Med. 152, 800–803.

Molema, J., van Herwaaraden, C.L.A., Folgering, H.T.M., 1989. Effects of long-term treatment with inhaled cromoglycate and budesonide on bronchial hyperresponsiveness in patients with allergic asthma. Eur. Respir. J. 2, 308–316.

Nieminen, M.M., Lahdensuo, A., Kellomaeki, L., Karvonen, J., Muittari, A., 1988. Methacholine bronchial challenge using a dosimeter with controlled tidal breathing. Thorax 43, 896–900.

Nijkamp, F.P., Vanderlinde, H.J., Folkerts, G., 1993. Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro. Am. Rev. Respir. Dis. 148, 727–734.

Persson, M.G., Gustafsson, L.E., 1993. Allergen-induced airway obstruction in guinea-pigs is associated with changes in nitric oxide levels in exhaled air. Acta Physiol. Scand. 149, 461–466.

Persson, M.G., Zetterström, O., Agrenius, V., Ihre, E., Gustafsson, L.E., 1994. Single-breath nitric oxide measurements in asthmatic patients and smokers. Lancet 343, 146–147.

Poullis, M., 1999. Mannitol and cadiac surgery. Thorac. Cardisvasc. Surg. 47, 58–62.

Rodwell, L.T., Anderson, S.D., Seale, J.P., 1992. Inhaled steroids modify bronchial responses to hyperosmolar saline. Eur. Respir. J. 5, 953–962.

Sanna, A., Kurtansky, A., Veriter, C., Stanescu, D., 1994. Bronchodilator effect of inhaled nitric oxide in healthy men. Am. J. Respir. Crit. Care Med. 150, 1702–1704.

Scott, J.A., Machoun, M., McCormack, D.G., 1996. Inducible nitric oxide synthase and vascular reactivity in rat thoracic aorta: effect of aminoguanidine. J. Appl. Physiol. 80, 271–277.

Silber, G., Proud, D., Warner, J., Naclerio, R., Kagey-Sobotka, A., Lichtenstein, L., Eggleston, P.A., 1988. In vivo release of inflammatory mediators by hyperosmolar solutions. Am. Rev. Respir. Dis. 137, 606–612.

Silkoff, P.E., McClean, P.A., Slutsky, A.S., Furlott, H.G., Hoffstein, E., Wakita, S., Chapman, K.R., Szalai, J.P., Zamel, N., 1997. Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide. Am. J. Respir. Crit. Care Med. 155, 260–267.

Tsoukias, N.M., George, S.C., 1998. A two-compartment model of pulmonary nitric oxide exchange dynamics. J. Appl. Physiol. 85, 653–666.