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Increased nitric oxide in expired air in patients with Sjögren's syndrome

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ABSTRACT: Nitric oxide has an important role in the regulation of airway function and can have pro-inflammatory effects. Bronchial hyperresponsiveness (BHR) and respiratory symptoms are common in patients with Sjögren's syndrome (SS). The aim of this study was to determine whether patients with SS have an increased amount of exhaled NO and whether this NO correlates with respiratory symptoms and BHR.

Exhaled NO was measured in 18 patients with SS and 13 normal subjects on three different occasions with intervals of at least 3 days using a chemiluminescence method. Airway responsiveness was assessed with methacholine provocation. Serum levels of myeloperoxidase (MPO), human neutrophil lipocalin (HNL), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) were measured.

Exhaled NO was significantly higher in patients with SS than in controls (147±82 *versus* 88±52 nL·min^{-1}; mean±SD; p=0.041). Exhaled NO was correlated with age (partial r=0.52, p=0.006) and serum HNL (partial r=0.46, p=0.014). There were no significant correlations between exhaled NO and respiratory symptoms, BHR or serum MPO, ECP or EPO. Disease duration was negatively associated with serum MPO (r=-0.47, p=0.043). In patients with SS, a positive correlation was found between symptom score and serum ECP (partial r=0.65, p=0.003) and EPO (partial r=0.62, p=0.004) and a negative correlation with age (partial r=-0.60, p=0.005).

In conclusion, elevated levels of exhaled nitric oxide in patients with Sjögren's syndrome were demonstrated. The mechanism underlying this increase in exhaled nitric oxide in Sjögren's syndrome is not known.

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It is now recognized that nitric oxide plays an important role in the regulation of airway function. Previously, NO was regarded as an atmospheric pollutant, but it is now considered useful in the diagnosis and monitoring of inflammatory airway diseases. Recently, NO excretion was found to be altered in certain inflammatory disorders. Asthmatic patients [1, 2] and patients with bronchiectasis [3] and respiratory infections [4] show increased orally exhaled NO, whereas patients with Kartagener's syndrome [5] and cystic fibrosis display a marked decrease in the NO exhaled *via* the nose [6].

Sjögren's syndrome (SS) is a chronic autoimmune inflammatory disease, characterized by lymphocytic and plasma cell infiltration, which mainly affects exocrine glands. SS can occur alone (primary SS) or in association with other rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (secondary SS). Several organs may be affected in SS, including the lungs. The respiratory manifestations include interstitial pneumonitis, "small airways disease" and pleuritis. The most common respiratory symptoms in SS are chronic dry nonproductive cough and dyspnoea, which affect about one-third of these patients [7]. Bronchial hyperresponsiveness (BHR) to methacholine has been observed in 60% of patients with primary SS [7, 8]. The mechanism underlying BHR in SS is not known, but may be related to bronchial inflammation.

The aim of this study was to determine whether patients with SS demonstrate an increased amount of NO in expired air, and whether the NO levels correlate with respiratory symptoms and BHR.

Patients and methods

Patients

Eighteen patients with primary SS who were being followed up as outpatients at the Deptartment of Rheumatology, Uppsala University Hospital, were studied (table 1). The diagnosis of SS was based on the Copenhagen criteria [9]. Each patient had keratoconjunctivitis sicca (shown by a pathological Schirmer's test (<10 mm in 15 min) and/or a short break up time (<10 s), and/or a positive Rose Bengal staining) as well as xerostomia (with a reduced unstimulated salivary flow rate of <0.7 mL·min^{-1} and/or a positive lower lip salivary gland biopsy and/or pathological salivary gland scintigraphy). Two abnormal tests were needed for a diagnosis of primary SS. The onset of the disease was 1–11 yrs (mean 8 yrs) prior to the

Table 1. – Characteristics of patients with Sjögren's syndrome and controls

	Sjögren's syndrome (n=18)	Controls (n=13)
Age yrs	54 (28–69)	30 (22–52)
Sex M/F	1/17	4/9
FVC % pred	94 (72–132)	95 (77–106)
FEV_1 % pred	93 (70–140)	95 (70–115)
sG_{aw} % pred	106 (18–430)	102 (59–223)
$FEF_{25\%}$ % pred	82 (41–148)	88 (46–157)
BHR %	55	15
Symptom score	2 (0–4)	0
Disease duration yrs	8 (1–11)	

Data presented as mean values with ranges in parentheses. M: male; F: female; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; sG_{aw}: specific airway conductance; $FEF_{25\%}$: forced expiratory flow at 25% of FVC; BHR: bronchial hyperresponsiveness.

study. None of the SS patients were current smokers. Twelve had never smoked and six were exsmokers (stopped smoking 4–17 yrs previously). Three of the SS patients used β_2-agonists as needed, and one used inhaled corticosteroids. Controls were subjects responding to a request for volunteers; none had asthmatic symptoms or was on antiasthmatic treatment (table 1). All were nonsmokers. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty of Uppsala University.

Study design

NO was measured on three occasions at least 3 days apart and within 3 weeks in each individual. At these visits, blood samples were taken for measurements of serum myeloperoxidase (MPO), human neutrophil lipocalin (HNL), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO), and lung function measurements were made. After the NO measurements, bronchial provocations were performed.

Questionnaire

All participants received a questionnaire concerning the occurrence of airway symptoms in the last 12 months. The questionnaire was based on the European Community Respiratory Health Survey questionnaire [10]. A symptom score (0–4) based on the number of respiratory symptoms in the last 12 months was calculated [11]. The airway symptoms included questions about wheeze, tightness in the chest and shortness of breath during the last 12 months.

Nitric oxide measurements

NO in mixed expired gas was measured over a 5-min period using a chemiluminescence NO analyser (model 42, Thermo Environmental Instruments Inc., Franklin, MA, USA). The system was calibrated with a mixture of NO in N_2 (AGA Gas AB, Lidingö, Sweden) at a concentration of 1 part per million (ppm). Hence, a four point calibration was performed (0, 10, 100, 1,000 parts per billion (ppb)). The calibration of the system was tested every morning and zero was set before each patient. The patient, with a nose clip in place, was connected to a three-way valve (Hans Rudolph, Inc., Kansas City, MO, USA) by a mouthpiece and inhaled synthetic NO-free air (AGA Gas AB) from a reservoir, and exhaled into a mixing box. The volume of the mixing box was 1.5 L and it was made of stainless steel. This material is known not to influence the NO measurements. NO samples were continuously drawn into the NO analyser from the mixing box. The exhaled volume was measured by a Wright spirometer. The amount of NO was calculated by multiplying the exhaled minute volume with the concentration of NO in the exhaled gas and then expressed in $nL \cdot min^{-1}$. NO values from the first minute were excluded and a mean value for each of the following minutes was obtained.

Measurements of serum MPO, HNL, ECP and EPO

Serum samples were obtained for the measurements of MPO, HNL, ECP and EPO. Whole blood was drawn into serum separation tubes (SSTs) and allowed to coagulate at room temperature for 60 min. Thereafter the serum was separated by centrifugation. The serum concentrations of MPO were assayed by means of a double antibody radioimmunoassay (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Radioimmunoassay of HNL was performed as described previously [12]. Serum ECP was measured by the Pharmacia CAP System^{TM} and serum EPO by a prototype immunofluorometric assay utilizing the Pharmacia CAP System. The MPO/HNL ratio was calculated as a marker of monocyte activity.

Lung function

Spirometry with measurements of lung volumes (forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and specific airway conductance (sG_{aw})) was performed before and after metacholine provocation with a Masterlab Trans spirometer and a Masterlab body plethysmograph (Erie Jaeger AG, Würzberg, Germany). The results were related to predicted values [13, 14].

Methacholine provocation

An automatic, inhalation synchronized dosimeter jet nebulizer, Spira Elektro 2 (Respiratory Care Centre, Hameenlinna, Finland) was used [15]. The bronchial provocations were performed from 08:30 h to 11:00 h. The subjects were instructed not to take any bronchodilating drug in the 8 h prior to a test. Increasing doses of methacholine chloride in a 0.9% saline solution were given until a decrease in FEV_1 of 20% or the highest concentration was reached. Methacholine was inhaled during five breaths, and lung function measurements followed in the next 3 min. Baseline values were obtained after inhalation of saline alone. The methacholine was given in 10 successive increasing doses ranging 0.0625–32 $mg \cdot mL^{-1}$. Patients with a provocative concentration of methacholine of <32 $mg \cdot mL^{-1}$ and causing a decrease FEV_1 ≥20% (PC_{20}) were diagnosed as having BHR.

Statistics

All data are presented as means±SD. In the analysis, the mean value of the three recordings of NO, inflammatory markers and lung function was used. Nonparametric tests, the Mann–Whitney U-test and the Spearman rank correlation coefficient were used to analyse data. Comparisons between groups were made with the Mann–Whitney U-test, and comparisons within groups with the Spearman rank correlation test. A multiple regression analysis was used to evaluate the independant relationship between inflammation markers, clinical data, increased exhaled NO and symptom score in patients with SS. In this analysis, the partial regression coefficient was calculated (partial r). Analysis of variance (ANOVA) for repeated measurements was used to analyse whether there were any significant interindividual differences in the amount of exhaled NO between the three measurements. A p-value of <0.05 was regarded as statistically significant.

Results

Exhaled nitric oxide in patients with SS and controls

The mean amount of exhaled NO was higher in patients with SS than in the control group (147±82 *versus* 88±52 $nL \cdot min^{-1}$; p=0.041) (fig. 1). No significant difference was found within subjects between the three different NO measurements either in SS patients or controls. The mean range of the NO measurements within subjects was 48±31 $nL \cdot min^{-1}$.

Exhaled nitric oxide and bronchial hyperresponsiveness

Ten (56%) of the 18 patients with SS and 2 (15%) of the 13 control subjects had BHR as judged by methacholine provocation (fig. 1). There was no significant correlation between mean exhaled NO and BHR in either group.

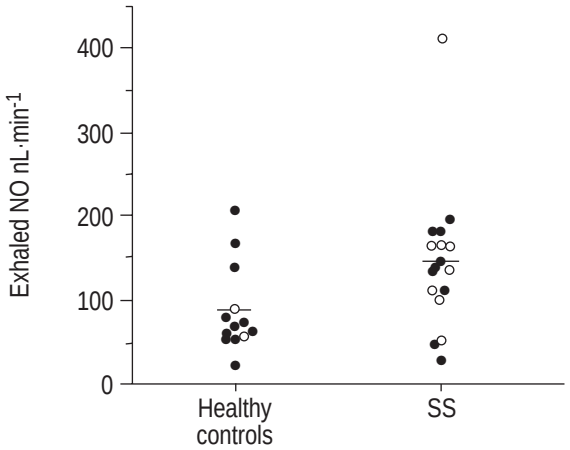

Fig. 1. – Mean exhaled nitric oxide in patients with Sjögren's syndrome (SS) and healthy controls with (○) and without (●) bronchial hyperresponsiveness. Bars represent mean values. The mean exhaled NO was significantly higher in patients with SS than healthy controls (p=0.041).

Exhaled nitric oxide and lung function

No significant difference in lung function was found between the two groups in FVC, FEV_1, sG_{aw} and forced expiratory flow at 25% of forced vital capacity (FEF_{25}) (table 1). There was no significant correlation between exhaled NO and lung function (FVC, FEV_1, sG_{aw}, FEF_{25}).

Symptom score

A positive correlation was found between symptom score and serum HNL (r=0.51, p=0.029), ECP (r=0.57, p=0.013) and EPO (r=0.53, p=0.024) in patients with SS. In a multiple regression analysis including age, disease duration, exhaled NO, serum HNL, MPO/HNL and EPO or ECP, symptom score was negatively correlated to age (partial r=-0.60, p=0.005) and positively correlated to serum EPO (partial r=0.62, p=0.004) and ECP (partial r=0.65, p=0.003) in the patients with SS.

Exhaled nitric oxide, age, disease duration and inflammation markers in serum

There was no significant difference in the tested inflammation markers (serum MPO, HNL, ECP and EPO) between the two groups. The MPO/HNL ratio was higher in patients with SS compared to the control group, although this difference was not statistically significant (p=0.06) (table 2).

The mean values for exhaled NO correlated significantly with age in the patients with SS (r=0.59, p=0.008) while no relationship between these variables was found in the control group. In a multiple regression analysis including age, disease duration, serum HNL, MPO/HNL and EPO, exhaled NO in patients with SS was correlated to age (partial r=0.52, p=0.006) and serum HNL (partial r=0.46, p=0.014). In the patients with SS there was a negative correlation with disease duration (yrs) and serum MPO (r=-0.49, p=0.039). There was no significant correlation between mean amount of exhaled NO and serum MPO, ECP and EPO in either group.

Discussion

In this study, a significant increase in exhaled NO was found in patients with SS compared to normal subjects. There was a positive correlation between the amount of exhaled NO and age and serum HNL in the patient group

Table 2. – Inflammatory markers in patients with Sjögren's syndrome and normal subjects

	Sjögren's syndrome (n=18)	Controls (n=13)
MPO $\mu g \cdot L^{-1}$	279±97	248±81
HNL $\mu g \cdot L^{-1}$	90±33	95±25
ECP $\mu g \cdot L^{-1}$	10±7	10±7
EPO $\mu g \cdot L^{-1}$	18±17	17±15
MPO/HNL	3.2±0.8	2.6±0.6

Data are presented as mean±SD. MPO: myeloperoxidase; HNL: human neutrophil lipocalin; ECP: eosinophil cationic protein; EPO: eosinophil peroxidase. Differences between values were nonsignificant.

that was not seen in the control group. However, exhaled NO did not correlate to airway hyperresponsiveness, respiratory symptoms or lung function.

Exhaled NO values in patients with SS were almost twice as high as those in healthy controls and, interestingly, of the same magnitude as values previously reported for patients with asthma [2]. The role of an increased exhaled NO in asthmatic patients is not clear, but it may be a protective mechanism to counteract bronchoconstriction [16].

The cellular origin of increased NO in patients with SS is not known. In asthmatic individuals, increased NO probably derives from inducible nitric oxide synthase (iNOS) activity in the airway mucosa. Immunohistochemical studies of bronchial biopsy specimens from asthmatic patients suggest that it may originate mainly from epithelial cells in the airways [17]. Many other cells in the respiratory tract are capable of producing NO, including macrophages, mast cells, neutrophils, endothelial cells, and vascular smooth muscle cells [18].

The elevated NO levels in patients with SS may derive from the epithelium, or derive from macrophages activated by cytokines released from lymphocytes. The third possibility is that NO is produced by inflammatory cells in the epithelium.

Four markers of inflammatory cell activity were measured in the present study. HNL is a newly recognized secretory protein of the secondary granules of neutrophil granulocytes. HNL seems to be entirely specific to neutrophils [12]. MPO is also found in the neutrophil granulocyte, but also to some extent in monocytes. Thus, the measurements of MPO may reflect the activity of both these cells and may be elevated in diseases with predominant monocyte activity. No ideal serum marker of monocyte activation is available. In the patients with SS, the ratio of serum levels of MPO/HNL was elevated, which suggests that the circulating monocytes are primed in these patients. In this study, two markers of eosinophils, ECP and EPO, were used. None of the proteins showed any elevation in the patients with SS. However, both eosinophil markers showed a positive correlation to respiratory symptoms while HNL was the only inflammation marker that correlated to exhaled NO levels. These findings may suggest the involvement of both eosinophils and neutrophils in the disease mechanism in these patients.

In this study, 56% of the patients with SS had BHR, a finding similar to that stated in other reports [7, 8]. The reason for the increased BHR in patients with SS is not clear. It is very likely that the dryness of the respiratory mucosa predisposes to increased bronchial responsiveness in these patients by increasing the osmolarity in the bronchial mucosa [19]. Other possible mechanisms underlying the BHR might be epithelial damage or an increased number of inflammatory cells in the airway mucosa.

No correlation was found between exhaled NO and BHR in patients with SS, although a previous study in asthmatic patients has demonstrated such an association [20].

Respiratory symptoms, mainly dry cough and dyspnoea, are common in patients with SS [7]. These symptoms have been related to dryness in the large airways secondary to lymphocyte infiltration of the glands of the tracheal mucous membrane. As in asthmatic patients, increased exhaled NO does not seem to correlate directly to respiratory symptoms [21].

In conclusion, this study has demonstrated elevated levels of exhaled nitric oxide in patients with Sjögren′s syndrome. The mechanism underlying this increase in exhaled nitric oxide is not known. Further studies comparing levels of exhaled nitric oxide and findings in bronchial biopsy specimens in Sjögren′s syndrome patients are required.

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