Research Communication

Flunisolide Decreases Exhaled Nitric Oxide and Nitrotyrosine Levels in Asthmatic Children

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Background. Exhaled nitric oxide (FeNO) has been reported to be elevated in the oxidative stress involved in asthmatic patients, and the reaction of nitric oxide (NO) with superoxide anions results in the formation of nitrotyrosine. The purpose of this study was to investigate the effect of inhaled steroid treatment on nitrotyrosine levels collected by exhaled breath condensate (EBC) and on FeNO. Methods. This was a single-blind placebo-controlled study. The lung function, FeNO, and nitrotyrosine levels were evaluated in 10 asthmatic children. Results. The nitrotyrosine levels were stable during the placebo period (T0 = 1.16 ng/ml versus T1 = 1.05 ng/ml; NS.), whereas they decreased after the treatment with flunisolide (T2 = 1.14 ng/ml versus T3 = 0.88 ng/ml; P < .001). No significant reduction in FeNO levels was observed after placebo treatment (T0 = 38.4 ppb versus T1 = 34.7 ppb, NS.). In contrast, FeNO values decreased significantly being at T3 = 14.9 ppb (T1 versus T3; P = .024). Conclusions. This study shows that corticosteroid treatment reduces nitrotyrosine levels in EBC of asthmatic subjects.

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INTRODUCTION

Oxidative stress is implicated in airway inflammatory disease such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) [1]. Different types of airway inflammatory cells, especially eosinophils, produce more superoxide anions (O2−) and release reactive nitrogen/oxygen compounds such as NO [2]. The reaction of NO and superoxide anions (O2−) in the airway results in the formation of peroxynitrite, a highly reaction oxidative species, which can nitrate the tyrosine residues of proteins to form the stable product of nitrotyrosine [3]. The formation of peroxynitrite is now well established in a variety of airway diseases [2, 3], and it has been demonstrated to be capable to damage pulmonary epithelial cells [4] and to induce airway hyperresponsiveness [5].

Exhaled NO and a large number of molecules collected by EBC have been demonstrated to be increased in the airway of asthmatic patients [6], and they are now established to represent markers of airway inflammation [7]. A significant correlation has been demonstrated between the reduction in FeNO levels and eosinophils markers of inflammation in asthmatic patients treated with inhaled corticosteroids [8]. A recent study suggests that inhaled steroid treatment resulted in a significant reduction to nitrotyrosine immunoreactivity in the airway epithelium, lung parenchyma, and inflammatory cells of patients with asthma [9].

The purpose of this pilot study was to investigate the effect of inhaled steroid treatment on nitrotyrosine levels, in comparison with pulmonary function and airway inflammation in asthma measured by FeNO.

MATERIALS AND METHODS

Subjects and experimental design

Ten children (6 males), ranging in age from 6 to 13 years with a history of mild-to-moderate bronchial asthma, according to the American Thoracic Society (ATS) definition [10] and positive skin prick tests to house dust mite (HDM) were evaluated. All children were recruited at the Department of Paediatrics, University of Verona, Verona, Italy. None of the patients had respiratory infections for at least two months before the beginning of the study and none of the children had received oral corticosteroids for at least two months before and after admission to the study.

The children did not present any clinically evident asthma exacerbation. The study was approved by the Local Ethics Committee and both children and their parents gave informed consent.
This was a single-blind placebo-controlled pilot study. All asthmatic children received placebo for 8 weeks (T0-T1) and then after a washout of 8 weeks (T1-T2), inhaled flunisolide (Lunibron A, Valeas SpA, Milano, Italy; 800 mcg/dose) for further 8 weeks (T2-T3). A final washout period of further 8 weeks without any treatment concluded the study (T3-T4).

During the study period the symptoms were recorded daily. Treatments were administered by the same model or pneumatic nebulizer (BimboNeb, Markos Mefar SpA, Brescia, Italy), which was provided to all of the patients.

Inhaled β2 agonists were allowed to be used as needed. Neither cromons nor inhaled steroids, other than flunisolide administered according to the experimental design, were allowed for the time of the study. None of the children received oral steroid after admission to the study.

The evaluations at T0, T1, T2, T3, and T4 consisted in lung function assessment, measurement of FeNO, and collection of EBC.

**EBC collection and measurement**

EBC samples were collected by a condensing device formed by two glass chambers (Incobar Srl, Modena, Italy) [11]. The inner glass chamber was cooled by means of ice and suspended in a larger glass chamber. The children were instructed to tidally breathe by the mouth through a two-way nonrebreathing valve for 15 min. To minimize salivary contamination the two-way valve served as a saliva trap, with a 12 cm banded tube vertically positioned between the mouthpiece and the condenser while the mouth of the subject remained at a lower position with respect to the inlet of the device. Children were asked to periodically swallow their saliva. EBC samples where stored in sterile tubes at −70 °C.

Nitrotyrosine levels were measured by specific enzyme immunoassay (EIA) kits (BIOXYTECH Nitrotyrosine—EIA; Oxis Research, Portland, Ore, USA) whose validity in EBC was shown by Hanazawa et al [2].

**Lung function and FeNO measurement**

Lung function was measured by a Vitalograph Compact spirometer (Vitalograph Ltd, Buckingham, UK); FEV₁ and FEF₂₅₋₇₅ were considered in the analysis of the results. The best value of three manoeuvres was accepted and expressed as percentage of the predicted normal values, according to ATS Guidelines [12].

FeNO was measured by chemiluminescence analyzer NIOX system (Aerocrine, Stockolm, Sweden), using a single-breath online method according to ERS/ATS Guidelines for FeNO measurement in children [13]. Children inhaled NO-free air and exhaled through a dynamic flow restrictor with a target flow of 50 mL/s for at least 6-7 s.

**Statistical analysis**

The data were expressed as means ± standard error of the mean (X ± SEM). The analysis of data showed normal distribution for all the investigated parameters in our study population. Differences between the times of the study (T0, T1, T2, T3, and T4) were performed by analysis of variance (one way RM ANOVA) and multiple comparison procedures by Bonferroni’s correction. Correlations were evaluated by simple regression test. A P value of < .05 was considered significant.

**RESULTS**

Two children presented an upper respiratory tract infection and they did not complete the study. These two subjects were excluded from the final analysis; therefore, 8 subjects completed the study and were considered in the final analysis. No adverse event was observed during the study period.

**FeNO**

Exhaled nitric oxide was measured in all asthmatic children. No significant reduction was observed in FeNO levels after the 8 weeks of placebo treatment (T0 = 38.4 ppb ± 4.53 versus T1 = 34.7 ppb ± 5.35, NS; 95% confidence interval CI, 27.6–49.1; P = .05) and after the first washout period (T1 = 34.7 ppb ± 5.35 versus T2 = 42.6 ppb ± 10, NS; 95% CI, 22–47.3; P = .05). In contrast, FeNO values decreased significantly being at T3 = 14.9 ppb ± 3.38 (T1 versus T3 P = .024; T2 versus T3 P < .001; 95% CI, 18.7–66; P = .05). After the second washout period (T4) there was a significant increase in FeNO levels to 45 ppb ± 7.31 (T3 versus T4P < .001; 95% CI, 6.8–22.8; P = .05) (Figure 1).

**Nitrotyrosine**

The quantitative determination of nitrotyrosine was possible in the EBC from all the subjects. The levels of nitrotyrosine in EBC were stable in the study group during the placebo and first washout period (T0 = 1.16 ng/mL ± .05, T1 = 1.05 ng/mL ± .03, T2 = 1.14 ng/mL ± .03; T0 versus T1 NS; T1 versus T2 NS), whereas they decreased after the period of flunisolide treatment (T3 = .88 ng/mL ± .04; T2 versus T3P < .001). After the second washout period...
represent mean values. The times of the study (T0, T1, T2, T3, and T4). Marked short lines
= (T4
the nitrotyrosine levels in EBC showed a significant increase
Figure 2: Individual data points of nitrotyrosine levels in EBC at
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nis established as a marker of airway inflammation [15],
tion, particularly in childhood asthma [14]. Exhaled NO is
oxides. Invasive methods until the discovery that exhaled nitric
This was a preliminary study investigating whether nitroty-
in the study group.
Lung function
All of the children performed lung function test. The data are
Correlations
No correlation was found between FeNO levels, nitrotyrosine
concentration, and spirometric parameters (FEV1, FEF25–75)
in the study group.
DISCUSSION
This was a preliminary study investigating whether nitroty-
rosine in EBC can reflect airway inflammation in asthma and
whether it was modulated by treatment with inhaled corti-
costeroids.
The investigating airway inflammation was mainly based
on invasive methods until the discovery that exhaled nitric
oxide can be used as surrogate marker of airway inflamma-
tion, particularly in childhood asthma [14]. Exhaled NO is
now established as a marker of airway inflammation [15],
it has been demonstrated to be able to predict lung func-
tion decline and to reflect the effect of treatment with anti-
flammatory agents [16] and also the present study shows
a significant decrease in FeNO levels when the children were
treated with inhaled flunisolide.
FeNO is, however, a single marker of airway inflam-
mation, and therefore for a more comprehensive evalua-
tion of the ongoing processes of inflammation in asthma
EBC has been recently proposed [17]. It has recently been
shown that EBC collected from adult patients contains a wide
number of molecules such as leukotrienes, prostaglandins,
albumin and other proteins, such as cytokines [6, 11,
17]. Furthermore, a number of mediators related to NO
pathway, including nitrate as a metabolite of nitric oxide,
nitrotyrosine, nitrosothiols in addition to small molecular
mediators associated with oxidative stress, such as hydrogen
ions and hydrogen peroxide, have been detected in EBC sam-
ples [2, 18].
Nitrotyrosine has been considered to be an indicator of
the involvement of reactive nitrogen species [3] and in the
airway inflammatory epithelium of asthma patients there
is a strong immunoreactivity to nitrotyrosine, suggesting a
pathophysiological role for reactive nitrogen species in
inflammatory lung diseases [19].
Recently a significant increase in nitrotyrosine levels in
EBC has been demonstrated in stable CF patients, compared
with normal subjects [18]. In addition, nitrotyrosine concen-
tration in EBC in mild and untreated asthmatic adults was
found to be correlated with exhaled NO level [2, 17].
Nevertheless, the results of the present study confirm the
findings of a recent report by Baraldi et al in asthmatic chil-
dren failing to show any correlation between FeNO and nitro-
tyrosine [20].
Furthermore, in the present study, it has been demon-
strated that exhaled nitrotyrosine concentrations in EBC was
decreased after inhaled steroid therapy. These data are in
agreement with the observation of Hanazawa et al [2] sug-
gesting that nitrotyrosine concentrations in EBC were signif-
icantly increased in steroid-untreated asthmatic patients.
The data from EBC samples confirm previous observa-
tions showing a direct effect of treatment with inhaled steroid
on nitrotyrosine-mediated immunoreactivity and NO pro-
duction in airway epithelium of asthmatic adult patients
has previously been demonstrated in samples obtained by
fiberoptic bronchial biopsies [9].
In addition, intense nitrotyrosine immunoreactivity was
demonstrated in the airway and parenchyma lung of asthma
patients who died of status asthmaticus despite steroid treat-
ment thus suggesting that status asthmaticus is characterized
by a failure of corticosteroids to control the formation of re-
active nitrogen species [19].
Furthermore, in this study, a significant increase in
FEF25–75 values during treatment with nebulized flunisolide
was observed, therefore suggesting an action of this drug
on peripheral airways. These data, indeed, are in agreement
with a recent observation by Bergeron et al who showed that
hydrofluoroalkane-flunisolide is associated with a significant
decrease in the expression of α-smooth muscle actin in pe-
ripheral airways, which correlated with improvement in pe-
ripheral airway function [21].
This was a pilot study, the main limitation for which was
the small sample size. However, despite the small number
of subjects, the statistical analysis showed highly significant
changes in the evaluated parameters for the actively treated
children. If, on the one hand, the small number of subjects
is likely to reduce the external validity of the study, on the
other hand, the level of statistical significance reached in such
a small sample size confirms the relevance of changes in the
investigational parameters.
In summary, this pilot study suggests that nitrotyrosine
concentrations, related to FeNO levels, were detectable in
EBC of asthmatic children and were reduced when the chil-
dren received corticosteroids treatment. In addition it also
provided evidence that nebulized flunisolide can have an
anti-inflammatory effect which is accompanied by a recovery of lung function values at the site of peripheral airways.

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Table 1: Mean (±SEM) lung function data and p values.

|         | T0      | T1      | T2      | T3      | T4      |
|---------|---------|---------|---------|---------|---------|
| FEV1 (% pred) | 86.5 ± 2.58 | 90.1 ± 2.88 | 86.4 ± 2.92 | 101.3 ± 4.81 | 84 ± 2.75 |
| FEV1 (%) | T0 versus T3 P < .001 | T1 versus T3 P < .001 | T2 versus T3 P < .001 | T3 versus T4 P < .001 | T4 versus T3 P < .001 |
| FEF25–75 (% pred) | 98.5 ± 7.73 | 101.1 ± 7.46 | 94.7 ± 6.45 | 121.9 ± 8.88 | 93.5 ± 5.13 |
| FEF25–75 (%) | T0 versus T3 P < .001 | T1 versus T3 P < .001 | T2 versus T3 P < .001 | T3 versus T4 P < .001 | T4 versus T3 P < .001 |