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Original Articles

Inhaled glucocorticosteroids decrease hydrogen peroxide level in expired air condensate in asthmatic patients

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H_2O_2 is elevated in the exhaled air condensate in several inflammatory disorders of the lung, including bronchial asthma, and thus may reflect inflammatory processes in the airways. Exhaled H_2O_2 may be used to guide the anti-inflammatory treatment of patients with asthma. Therefore in this study we analysed the effect of inhaled glucocorticosteroid beclomethasone for 4 weeks on H_2O_2 level in the exhaled air condensate.

Seventeen asthmatics and 10 healthy subjects were included to the study. Eleven patients were given inhaled beclomethasone and six were given placebo (3M Health Care). In all patients pulmonary function tests were performed. H_2O_2 in the expired air condensate was measured spectrofluorimetically (homovanillic acid method).

Inhaled beclomethasone significantly decreased H_2O_2 in the expired air condensate in the active-treatment group, with a fall from baseline on day 1 which remained on day 43 (follow-up) ($P<0{\cdot}05$). Exhaled H_2O_2 in the active-treatment group was significantly lower than that in placebo group ($P<0{\cdot}05$). A negative correlation between H_2O_2 and forced expiratory volume in 1 sec (FEV_1) on day 29 was observed.

The decrease in exhaled H_2O_2 in the active-treatment group was accompanied by an improvement in pulmonary function tests results.

Inhaled glucocorticoids reduce the level of H_2O_2 in the expired air condensate of asthmatic patients over a 4-week period and this may reflect their anti-inflammatory activity in lung diseases.

Key words: bronchial asthma; airway inflammation; hydrogen peroxide; expired air; breath condensate; inhaled glucocorticosteroids.

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Introduction

An important feature of bronchial asthma is the influx of circulating phagocytes, including eosinophils, mast cells and neutrophils, into the bronchial wall (1–3). After activation they generate reactive oxygen species, including hydrogen peroxide (H_2O_2), which can play an important role in the development of lung damage and bronchial hyperresponsiveness (4–8). It has been proved that neutrophils and eosinophils from both adult and children asthmatic patients can generate increased amounts of $O_2^{\bullet-}$ and H_2O_2 after challenge with phorbol esters and fMLP than cells of matched healthy subjects (9–12). Moreover, the ability of neutrophils isolated from asthmatics to produce superoxide anion correlated with the degree of airway hyperresponsiveness to inhaled metacholine and histamine (13). Furthermore, in asthematic children, the generation of $O_2^{\bullet-}$ was at a significantly higher rate in children who suffered attacks than those who did not (13). This is consistent with the observation that patients with exacerbated asthma exhale more H_2O_2 than patients with stable disease (14). H_2O_2 in the expired breath condensate can be elevated in other lung inflammatory diseases — such as chronic obstructive pulmonary disease (COPD), and adult respiratory distress syndrome (ARDS) (15,16).

In asthmatic patients an increased level of H_2O_2 in the expired breath condensate is positively correlated with an increased level of lipid peroxidation products (thiobarbituric acid reactive species) in the expired breath condensate which proves oxidant/antioxidant imbalance in the airway of these patients (17). An intense airway inflammation can be caused either by H_2O_2 alone or a newly generated hydroxyl radical (18).

Phagocytes are an important source of H_2O_2. It is released in extracellular fluid and in the airways. H_2O_2

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which has not been decomposed by antioxidant enzymes can be excreted with expired air. Topical glucocorticosteroids are highly effective in the control of asthma and they are now the first-line therapy in patients with persistent symptoms. Their effectiveness in controlling asthma is medicated by inhibiting the inflammatory process through influence on inflammatory mediators (20–23). The induction of inducible nitiric oxide synthase can be inhibited by glucocorticosteroids (24). This is consistent with studies of Kharitonow *et al*. who have proved that inhaled glucocorticosteroids decrease nitric oxide in the exhaled air of asthmatic patients (25). Steroids markedly reduce the survival of inflammatory cells such as eosinophils by inducing apoptosis (26). The eosinophil count in the induced sputum of asthmatic children is positively correlated with the NO level in expired air and the reduction of survival rates of these cells could be responsible for the NO decrease in expired air. Collection of the exhaled air condensate provides a non-invasive method of obtaining samples from the lower airways (27,28). Exhaled H_2O_2 may be used instead as a diagnostic test to guide anti-inflammatory treatment. Until now there has been no double-blind, randomized clinical trial on the effect of inhaled steroids on H_2O_2 level in expired air of asthmatic patients. We have therefore studied the effect of inhaled beclomethasone dipropionate at a low dose on the level of H_2O_2 in the expired air condensate of steroid-naive asthmatic patients.

Materials and Methods

STUDY POPULATION

Our study was associated with a 6 week, randomized, double-blind, double-dummy, multicentre study on efficacy of 400 μg day^{-1} chlorofluorocarbon-free beclomethasone dipropionate, delivered as an extrafine aerosol in adults with moderate asthma approved by the sponsor (3M pharmaceuticals, Loughborough, England) and Local Ethics Committee as safe and non-invasive. It included 17 asthmatic subjects (mean age 40 $\pm$ 9 years., 11 males and five females) who had not suffered from any infectious disease for the last 4 months (Table 1). Eleven patients were given inhaled HFA-beclomethasone dipropionate 400 μg day^{-1} or HFA-placebo. They were asked to visit the Clinic on day 15, 29 and fulfil the follow-up visit at day 43 (Fig. 1). At all visits lung function tests and collection of breath condensate were performed. The drug dose used in this study is lower than that recommended in current treatment guidelines for moderate asthma, however we used reformulated CFC-free propellant, hydrofluoroalkane 134a (HFA), resulting in an extra fine aerosal which gives improved drug delivery to the airways. The study of Matthys has provided data that a daily dose of 400 μg HFA-beclomethasone (3M Pharmaceuticals, Loughborough, England) provides effective control in patients with moderate asthma (29). They were recruited from the Medical University Out-patient Clinic register. Asthmatic subjects were asked to stop any medication except short acting β-agonists (salbutamol or fenoterol) and to come to the clinic after a 4 week washout period to perform lung function tests. Bronchial asthma was diagnosed on history of wheezing dyspnoea and previous documentation of bronchodilator-induced bronchial reversibility measured as more than a 15% increase of FEV_1 and the presence of airway hyper-reactivity after histamine challenge test with PC_{20} of less than 8 mg ml^{-1} according to the method of Cockroft (30).

Pulmonary function FEV_1 between 50% and 80% of predicted value, and bronchial reversibility of at least 15% were basic inclusion criteria. The other inclusion criteria were the ability to stop therapy other than β_2-agonist therapy. The duration of bronchial asthma was 1–18 years, mean 7 $\pm$ 5 years. Nine subjects were atopic as revealed by the presence of immediate positive response to more than 12 common aeroallergen extracts (Allergopharma, Joachim Ganzer KG Reinbeck/Hamburg, Germany) in Poland. Seven asthmatics had a positive skin prick test to house dust. The criteria for definition of non-atopic status was the absence of immediate response on prick test and no family history of atopy. Spirometry was performed with Flowscreen (Erich Jaeger GmbH&Co. Germany) equipped with software compatible to American Thoracic Society standards (31). None of the women were pregnant as assayed by urine pregnancy test (Clearview HCG, Unipath GmbH Wesel, Germany) or took oral contraceptives. This study was approved by the local Ethics Committee and informed consent was obtained.

TABLE 1. Characteristics of study population

	Active treatment patients	Placebo patients	Healthy control
Number	11	6	10
Age (years)	41·4 $\pm$ 9	37·3 $\pm$ 8	43·5 $\pm$ 5
Sex (M:F)	9:2	2:4	6:4
FVC%	96·5 $\pm$ 1·2	92·5 $\pm$ 6·8	97·5 $\pm$ 6·7
FEV_1%	69·3 $\pm$ 3·2	67·1 $\pm$ 4·2	94·9 $\pm$ 5·1
FEV_1 reversibility (%)	28·4 $\pm$ 11	27·3 $\pm$ 8	2·9 $\pm$ 4·3
Asthma duration (years)	6 $\pm$ 9	7 $\pm$ 5	—

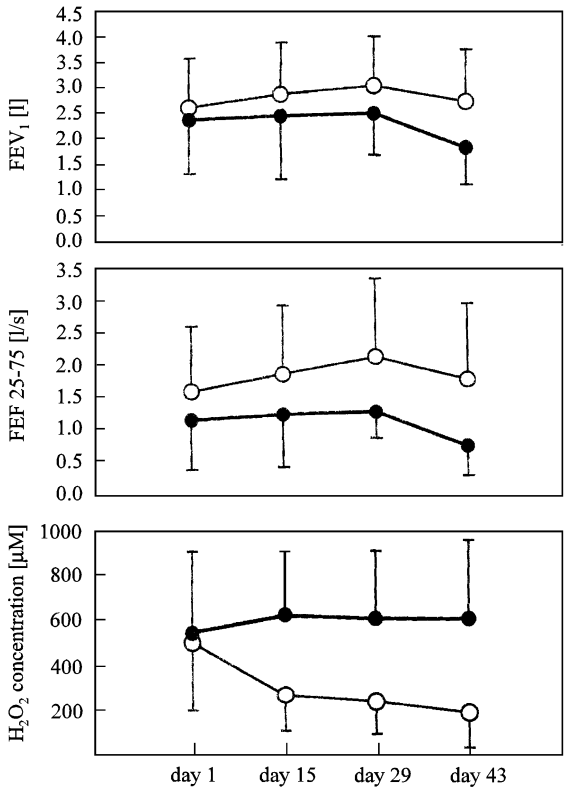

FIG. 1. Increase in FEV_1 in active-treatment group (○) compared with placebo (●). Median FEV_1 at day 43 was significantly higher in HFA-beclomethasone treatment group compared with placebo ($2{\cdot}32 \pm 0{\cdot}78$ *vs.* $1{\cdot}56 \pm 0{\cdot}10$ l) ($P < 0{\cdot}05$). Increase in FEF_{25-75} in the active-treatment group (○) compared with placebo (●). In active treatment group FEF_{25-75} on 43 was significantly higher than FEF_{25-75} in placebo group ($1{\cdot}69 \pm 0{\cdot}92$ *vs.* $0{\cdot}71 \pm {\cdot}20$ l/sec, $P < 0{\cdot}05$). Changes in exhaled H_2O_2 in HFA-beclomethasone group (○) and placebo-patients (●). The fall from $0{\cdot}57 \pm 0{\cdot}25$ µmol on day 1 of the study to $0{\cdot}33 \pm 0{\cdot}10$ µmol on day 29 ($P < 0{\cdot}05$). The concentration of exhaled H_2O_2 in active-treatment group remained stable on treatment days (1–29) and follow up (day 43) and was significantly lower than that in placebo group on all days ($P < 0{\cdot}05$).

COLLECTION OF AIR CONDENSATE

The air condensate was collected in a tube installed in the polystyrene foamed container filled with ice and salt as previously described. At the end of collection 2–5 ml aliquots of condensate osmolality using an osmometer 800cl, Trident (Warsaw, Poland). The rest was stored at $-80°$C for not longer than 7 days until H_2O_2 measurement. Our previous experiments have shown that samples of expired breath condensate and 7×10^{-1} M H_2O_2 solution, under these conditions, remain stable after 14 days of storage. Similarly, 5 nM H_2O_2 incubated in the device for 20 min at 0°C revealed no significant changes of ability to react with homovanillic acid (28). All collections were performed between 9 and 11 am and patients were asked to stop any medication 12 h before the visit.

MEASUREMENT OF HYDROGEN PEROXIDE

The content of H_2O_2 in expired breath condensate was determined according to the method of Ruch *et al.* (32). Briefly, 600 µl of expired breath condensate was mixed with 600 µl solution of HRP (1U ml^{-1}) containing 100 µM homovanillic acid and incubated for 60 min at 37°C. Afterwards the sample was mixed with 150 µl 0·1 M glycine–NaOH buffer (pH 12) with addition of 25 mM EDTA and transferred into microcuvette (PE 5200-4339). The homovanilic acid oxidation product as a measure of the amount of H_2O_2 was determined spectrofluorimetically using Perkin Elmer Luminescence Spectrometr LS-50 (Norwalk, CT) operating in the read mode. Slit widths were set at 10 nm for both emission and excitation and the integrate time was 0·1 sec excitation was at 312 nm and emission was measured at 420 nm. Readings were converted into nmol using regression equation $Y = (x\text{-}x_0)67{\cdot}64$ (Y-nmol of H_2O_2 per one sample of expired breath condensate, X-intensity of emission at 420 nm expressed in arbitrary units, x_0-intensity of emission given by reference sample containing distilled water instead of breath condensate) obtained fron three series of calibration experiments with 19 increasing (0·0125–25 µM) H_2O_2 concentrations. The confidence level was 95% and the *P*-value was less than 0·03 and 0·0001 for the constant and regression coefficient, respectively. The linear least square estimation was used for calculation of the regression equation. The lower limit of H_2O_2 detection was 83 µM and the calibration curve was linear up to a concentration 16·7 µM H_2O_2. Values of H_2O_2 concentrations obtained in this study are different to those in our previous work, in which we were using different sample volumes and the concentrations were expressed in nmoles per sample (17,29).

SATISTICAL ANALYSIS

Data obtained in this study were non-parametric and therefore are expressed as the median ± interquartile range. Readings that gave results below the method sensitivities (83 nmol H_2O_2) were assumed to be 0 nmol. The differences between results in group of placebo and active-treatment subjects were determined by Mann–Whitney analysis based on rank sums. The median FEV_1, FEF_{25-75} and concentrations of hydrogen peroxide between days were compared using Mann–Whitney *U* test. A *P*-value less than 0·05 was considered to be significant. A rank correlation and pulmonary function tests. All calculations were performed using Microsoft Excel Version 5·0 software.

Results

H_2O_2 was detected in 10 active-treatment patients and in five patients in the placebo group. In one patient in each

group no H_2O_2 in the expired air condensate was detected. The baseline exhaled H_2O_2 was significantly higher in asthmatic patients than in normal subjects ($0{\cdot}54 \pm 0{\cdot}21\,\mu M$ *vs.* $0{\cdot}03 \pm 0{\cdot}02\,\mu M$, $P < 0{\cdot}01$) and this is consistent with our previous results (17). There were no statistically significant differences in H_2O_2 concentration in exhaled air condensate nor pulmonary function tests between active-treatment and placebo group just before randomization on day 1.

Inhaled HFA-beclomethasone significantly decreased exhaled H_2O_2 in active treatment group, with a fall from $0{\cdot}57 \pm 21\,\mu M$ on day 1 of the study to $0{\cdot}18 \pm 0{\cdot}15\,\mu M$ on day 29 ($P < 0{\cdot}05$) (Fig. 1). The biggest fall was observed between day 1 and day 15 of the study ($0{\cdot}57 \pm 0{\cdot}25\,\mu M$ *vs.* $0{\cdot}23 \pm 0{\cdot}11\,\mu M$, $P < 0{\cdot}05$). The concentration of exhaled H_2O_2 in active-treatment group remained decreased on treatment days (1–29) and follow-up and was significantly lower than that in placebo group ($0{\cdot}23 \pm 0{\cdot}11$ *vs.* $0{\cdot}66 \pm 0{\cdot}19\,\mu M$ on day 15, $0{\cdot}33 \pm 0{\cdot}1$ *vs.* $0{\cdot}69 \pm 0{\cdot}26\,\mu M$ on day 29, and $0{\cdot}18 \pm 0{\cdot}15$ *vs.* $0{\cdot}69 \pm 0{\cdot}21\,\mu M$ on day 43, respectively, $P < 0{\cdot}05$). There were no significant differences between males and females.

In the placebo group H_2O_2 remained stable over the study with no statistically significant differences between days 1, 15, 29 and 43. However, there can be observed a big variability in particular patients with no detectable H_2O_2 level at day 1 and relatively high H_2O_2 concentration on days 15 and 29. In some patients a big variability in H_2O_2 concentration was observed, too. A negative correlation was found between H_2O_2 in exhaled air concentration on day 29 and FEV_1 on that day in active treatment group ($r = -0{\cdot}63$, $P < 0{\cdot}05$) (Fig. 2).

The decrease in H_2O_2 concentration in expired breath condensate was accompanied by an increase in pulmonary function test results, however they were not statistically significant. Median FEV_1 on day 43 was significantly higher in HFA-beclomethasone treatment group compared with that in placebo group ($0{\cdot}32 \pm 0{\cdot}78$ vs. $1{\cdot}56 \pm 0{\cdot}10$ l) (Fig. 1). Similarly, in active treatment group FEF_{25-75} on day 43 was significantly higher than FEF_{25-75} in placebo group ($1{\cdot}69 \pm 0{\cdot}0.92$ *vs.* $0{\cdot}71 \pm 0{\cdot}20$l/sec, $P < 0{\cdot}05$) (Fig. 1).

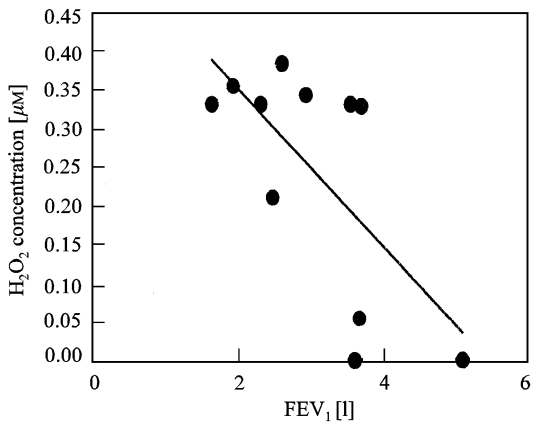

FIG. 2. Negative correlation between H_2O_2 concentration in expired air and FEV_1 on day 29 in active treatment group ($r = -0{\cdot}63$, $P < 0{\cdot}05$).

Discussion

We have demonstrated that an inhaled glucocorticoid significantly reduces the level of H_2O_2 in the expired air condensate of asthmatic patients over a 4-week period and that the decreased H_2O_2 concentration remains stable for 2-week follow-up. These findings are compatible with the hypothesis that the increase in exhaled H_2O_2 is due to airway inflammation. H_2O_2 has been accepted as a marker of airway inflammation as it can be produced by inflammatory cells involved in mucosal inflammation in asthmatic subjects (2,3). Moreover, stimulated macrophages from asthmatic patients produce more reactive oxygen species than those from healthy subjects (33). H_2O_2 is elevated in expired air condensates in various inflammatory lung disorders such as ARDS, acute hypoxaemic respiratory failure, cigarette smoking and COPD. Dohlman described an increased H_2O_2 level in expired air condensate in a relatively small group of asthmatic children, mainly in those with acute disease. In some of them glucocorticosteroids were administered and this resulted in decreased exhaled H_2O_2 level (14). It is postulated, however that inflammation may be present in symptom free asthmatics (34) and as a result of this phenomenon elevated H_2O_2 in stable asthmatics could be observed (35). Jobsis has described in an open study a group of 66 children with stable asthma, 41 of whom were receiving inhaled cortiosteroids daily. There was a significant difference in median H_2O_2 concentration between asthmatics without anti-inflammatory treatment and healthy controls ($P < 0{\cdot}05$). The difference in median H_2O_2 concentration in the corticosteroid group was smaller, pointing out the association of inhaled steroid with lower exhaled H_2O_2 concentration in asthmatics. A study in ARDS patients treated with corticosteroids showed a tendency towards lower levels of H_2O_2 in the expired air condensate as compared to steroid-naive ARDS patients (36).

We speculate that there are several mechanisms whereby inhaled glucocorticosteroids may reduce exhaled H_2O_2. Steroids inhibit the synthesis of pro-inflammatory cytokines in inflammatory cells such as macrophages (20). This could lead to decreased activity of NADH oxidase which is speculated to be the main source of H_2O_2 in asthmatic patients. It was proved that neutrophils from both adult and children asthmatic patients can generate increased amounts of $O_2^{\bullet -}$ and H_2O_2. A close relationship between the opsonized zymosan-induced chemiluminescence of neutrophils and bronchial hyper-reactivity to histamine in asthmatic children can also be observed (13). Inhibition of the H_2O_2-generating system by inhibiting the synthesis of pro-inflammatory cytokines could be responsible for decreased H_2O_2 level in exhaled air of asthmatic subjects. Finally, steroids may reduce the number of activated cells that release pro-inflammatory cytokines in the airways by blocking their influx into the lung (26).

In this study we observed an improvement in lung function test over the study, especially in small airways. A negative correlation was found between H_2O_2 concentration in exhaled air on day 29 and FEV_1 in active treatment group. The bigger FEV_1 increase the bigger H_2O_2 fall in

expired air. This could reflect anti-inflammatory action of inhaled steroid. The smaller inflammation in the airways is accompanied by lower H_2O_2 concentration in expired air and improvement in pulmonary function results.

What is interesting is that pulmonary function tests in the active treatment group were higher than the placebo group but not statistically significant. This can be due to a small population who completed H_2O_2 collection and finally were included into our part of the whole trial. However, it was proved in the whole study population that inhaled HFA-beclomethasone dipropionate 400 μg day^{-1} gives improved drug delivery to the airways and that active treatment produces significant improvements compared with placebo in evening, PEF, FEV_1 and FEF_{25-75} thus providing an efficient control of patients with moderate asthma (29). A relatively low dose of anti-inflammatory medication was able to significantly decrease H_2O_2 level in expired air. As H_2O_2 is elevated in several lung inflammatory disorders reflecting an oxidant overload in the airways; rather than a diagnostic test, exhaled H_2O_2 may be used to monitor anti-inflammatory treatment and estimate disease severity over time within subjects.

This is the first double-blind, placebo-controlled study investigating the effect of an inhaled glucocorticosteroid on H_2O_2 level in expired air condensate. It is difficult to demonstrate a clear dose-response effect of glucocorticosteroid in asthma and many patients are needed for such studies because of the variability in the response between different patients (37). It is possible that using H_2O_2 in exhaled breath condensate as a dose-response to inhaled steroids may be documented as a means of monitoring anti-inflammatory action of the steroid additionally to measurement of airway responsiveness or pulmonary function, and thus may provide a non-invasive means to monitor the control of airway inflammation in asthmatic patients. However large fluctuations in levels of H_2O_2 occur within individual subjects and therefore this might limit its value.

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