Markers of inflammation and oxidative stress in exacerbated chronic obstructive pulmonary disease patients

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Summary COPD is characterised by damage to small airways due to an inflammatory process as well as an imbalance between oxidants and antioxidants. Several cytokines and cell adhesion molecules enhancing a mainly neutrophilic inflammation have been associated with COPD. The aim of the study was to investigate whether inflammation or oxidative markers gave an indication of the course of COPD during an exacerbation.

Fourteen patients with moderate to severe COPD admitted to the St. Antonius Hospital because of an exacerbation have been monitored during treatment with prednisolone 50 mg intravenously during 24 h at admission, reduced to 25 mg at day 3 and tapered off with oral prednisolone at day 7. On three separate occasions, day 1, 3 and 7, H2O2 in exhaled air, IL-8 and the soluble cell adhesion molecule sICAM and sE-selectin in serum were measured.

We compared the patients at day 1 with healthy controls (in both non-smokers and smokers). Furthermore, we examined the changes from the study group in time during therapy. At admission all the markers were raised in comparison with the control groups. During treatment H2O2 concentrations in breath condensate declined significantly (P < 0.001) as well as IL-8 and sICAM in serum (P = 0.002, respectively, P < 0.001). There was no significant change in sE-selectin (P = 0.132). No significant improvement has been found in spirometry.

These data suggest that the markers H2O2 in exhaled air, IL-8 and sICAM in serum are suitable markers in monitoring exacerbated COPD.

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Introduction Chronic obstructive pulmonary disease (COPD) has an increasing prevalence with high morbidity and mortality. COPD intensity can vary in time; the forced expiratory volume in 1 s (FEV1) declines
progressively while smoking, recurrent exacerbation and occupational or environmental pollution accelerates this process.\(^1\)

Airway inflammation and oxidative stress have been implicated in the pathogenesis of COPD. The inflammation in COPD shows high numbers of neutrophils, macrophages and T-lymphocytes, especially of the CD8 positive T cell subset in bronchoalveolar lavage fluid.\(^2,3\) Induced sputum revealed elevated levels of tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-8 (IL-8).\(^4,5\) IL-8 has been detected in plasma of patients with COPD during an acute phase response.\(^6\) IL-8 is a strong chemoattractant of neutrophils, it also initiates degranulation, production of reactive oxygen species (ROS) and increased expression of cell adhesion molecule CD1b/CD18 (MAC-1).\(^7,8\) Cell adhesion molecules are major factors in recruitment, cumulating and site-specific activation of inflammatory cells in the airways.\(^9–11\) An increased expression of E-selectin on endothelium and ICAM-1 on endothelium as well as on epithelial surface has been associated with COPD.\(^12,13\)

Oxidative stress also plays an important role in the pathogenesis of COPD. Oxidative stress is caused by an imbalance between the production of oxidants and the presence of antioxidants. Oxidants are produced in the lungs by inflammatory cells, especially neutrophils and macrophages. There is strong evidence of an increased burden of oxidants in the lungs of patients with stable COPD.\(^14,15\) During an exacerbation the production of ROS is increased presumable because of a large burden of activated inflammatory cells in the lower airways as a result of release of cytokines and upregulation of cell adhesion molecules.\(^7\)

An important exogenous source of oxidants in COPD patients is cigarette smoke. An excess of ROS can directly damage lung cells by oxidation of lipid membranes, proteins and DNA. Besides, abundant ROS cause inactivation of \(\alpha_1\)-antiprotease.\(^16\)

The level of oxidative stress may be used as an indirect marker of airway inflammation. It can be determined by measuring hydrogen peroxide (H\(_2\)O\(_2\)) in exhaled breath condensate, a simple and non-invasive procedure.\(^17–24\) Several investigators found significant increased H\(_2\)O\(_2\) levels in patients with stable COPD compared to healthy non-smoking and an additional increase in COPD patients with an acute exacerbation.\(^18,19,23,24\)

Our study aimed to investigate H\(_2\)O\(_2\) in exhaled air, IL-8 and the soluble cell adhesion molecules sICAM-1 and sE-selectin in serum as suitable markers for monitoring chronic obstructive pulmonary disease during treatment of an exacerbation with prednisolone.

Patients and methods

Patients

Fourteen patients with a moderate to severe COPD (FEV\(_1\) < 70% predicted, FEV\(_1\)/FVC < 70% and reversibility to bronchodilators less than 10% of the predicted value), admitted to the St. Antonius Hospital because of an exacerbation, participated the study. There were nine men and five women, mean age 63.6 years (54–78). The diagnosis COPD was made according to ATS standards.\(^1\) All subjects had a history of smoking (> 30 packyears), four were current smokers.

At admission there were no clinical signs of respiratory bacterial tract infection or inflammation elsewhere. Before admission all except two patients inhaled corticosteroids, six had a low maintenance dose oral prednisolone (7.5–10 mg) and all were treated with inhaled short and/or long acting \(\beta_2\)-agonists, 11 patients had also received anticholinergic inhalation therapy. Seven patients used N-acetylcysteine and eight patients used slow-release theophylline on regular base.

Patients with pulmonary disorders at present or in the past other than COPD (e.g. tuberculosis, bronchiectasis and asthma) were excluded.

All patients gave written informed consent to participate in the study, which was approved by the local Ethics Committee of the hospital.

Control group

The control group consisted of 15 healthy smoking volunteers, eight men and seven women, mean age 44.4 years (20–57) and 15 healthy non-smoking volunteers, six men and nine women, mean age 46.0 years (21–59) with normal spirometric test [forced expiratory volume in 1 s (FEV\(_1\)) and inspiratory vital capacity (IVC)] and no signs of infectious disease during the past 4 weeks. All smoking controls had a history of more than eight pack-years. The non-smokers never smoked.

Study design

The samples were taken during 7 days before the patient had breakfast between 8.30 and 9.30 a.m.; on three separate occasions, day 1 (before or shortly after start treatment), day 3 and day 7.

At admission all patients were treated with 50 mg prednisolone during 24 h; after 2–3 days the dose was reduced to 25 mg during 24 h and at day 7 all subjects except one started a scheme of oral prednisolone once a day. Theophylline as well as
N-acetylcysteine were continued if previously used and inhalation with $\beta_2$-agonists and anticholinergics was intensified. None of the patients smoked cigarettes during admission.

On three separate occasions, day 1 (before or shortly after start treatment), day 3 and day 7 (during treatment) spirometric tests were performed. Within 60 min after the spirometric test the collection of $\mathrm{H}_2\mathrm{O}_2$ in exhaled breath condensate and IL-8, sICAM-1 and sE-selectin in serum was performed.

**Collection and measurement of exhaled $\mathrm{H}_2\mathrm{O}_2$**

Before collection a mouthwash was performed using 15 ml chlorohexidine (0.2%) during 1 min to eliminate contamination of expired breath by mouth flora. The exhaled breath condensate samples were taken between 8.30 and 9.30 a.m. The patient was spontaneously breathing through a mask with a two way valve (Downs CPAP Mask, Vital Signs Inc., USA) for a 20 min period. The expired breath condensate was collected by passing the exhaled air through a specially constructed collecting device which consist of a 140 cm cold finger with an internal diameter of 15 mm. At the end of the coldfinger, the condensate was collected into a small glascontainer. This device was placed in a polystyrene foam container filled with ice, water and salt (−2°C to −5°C). A second collecting device was coupled to the first device to be sure that all the exhaled breathe did indeed condensate. After collection the condensate was immediately transported to the laboratory in a light protected vial and stored at −70°C and processed within 7 days. The device was cleaned with distilled water each time before and after the measurement.

$\mathrm{H}_2\mathrm{O}_2$ concentrations were measured in expired breath condensate of the patients as described by Gallati and Pracht. Two hundred microlitre breath condensate or standard $\mathrm{H}_2\mathrm{O}_2$ (Sigma-Aldrich Company, Milwaukee, USA) was incubated in a microtiterplate well with 20 $\mu$l 1.155 mmol/l 3,3′,5,5′-tetramethylbenzidin (Sigma-Aldrich Company, Milwaukee, USA) in 1.155 mol/l citric acid buffer pH 3.95 and 10 $\mu$l 52.5 U/ml horseradish peroxidase (Sigma-Aldrich Company, Milwaukee, USA) for 25 min in the dark at room temperature. Subsequently the mixture was acidified to a pH of 1 with 10 $\mu$l of 18N sulphuric acid. The reaction product was measured spectrofotometrically at 450 nm using an automated Benchmark microplate reader (Bio-Rad Laboratories, Hercules, USA). The absorbance is directly proportional to the concentration of $\mathrm{H}_2\mathrm{O}_2$.

The detection limit of $\mathrm{H}_2\mathrm{O}_2$ in this method is 0.11 $\mu$mol/l. The intra-assay variability are 13.3%, 6.7% and 2.3% for standard solutions of 0.1, 0.25 and 0.5 $\mu$mol/l $\mathrm{H}_2\mathrm{O}_2$ ($n=10$), respectively. The stability of $\mathrm{H}_2\mathrm{O}_2$ in breath condensate is 10 days with an inter-assay variability over time, to check the storage of $\mathrm{H}_2\mathrm{O}_2$ in breath condensate at −80°C, from 3.7% ($n=5$, day 1, 4, 7, 10).

**IL-8 assay:** The IL-8 is a solid-phase, two-site chemiluminescent immunometric assay measured on the Immulite analyser (DPC, Los Angeles, USA). The detection limit of IL-8 is 2.0 ng/l (duplicate CV of the control group 5.4%; $n=10$) with a reference value of <8.9 ng/l.

**Human soluble ICAM-1 and E-selectin assay:** Both tests are solid phase enzyme-linked immunosorbert assays based on the sandwich principle (Hycult biotechnology, Uden, The Netherlands) measured on a automated microplate reader (Benchmark, BIO-RAD, Veenendaal, The Netherlands). The detection limit of ICAM-1 is 0.10 $\mu$g/l (duplicate CV of the control group 3.6%, $n=10$) with a reference values of 39–165 $\mu$g/l and 0.05 $\mu$g/l for E-selectin. The IL-8 is a solid-phase, two-site chemiluminescent immunometric assay measured on the Immulite analyser (DPC, Los Angeles, USA). The detection limit of IL-8 is 2.0 ng/l (duplicate CV of the control group 5.4%; $n=10$) with a reference interval of <27.2 $\mu$g/l for E-selectin.

**Lung function measurements:** FEV$_1$ and vital capacity (IVC) were measured using a dry spirometer (MasterScreen, Jaeger Benelux, Amsterdam, The Netherlands). Values were expressed as percentage of predicted according to the ATS-standards.

**Sputum sampling and culture:** At admission sputum was sampled in all patients. The investigation of sputum was performed on an aliquot of a fresh obtained spontaneous sputum sample by an experienced microbiology technician and according to its predominant macroscopic appearance judged as mucoid or purulent. Direct examination using the Gram stain technique was applied to judge the quality of the sputum. If the quality of the sputum was sufficient a culture was made, and the number of bacteria in the sputum was semi-quantitatively assessed after culture appears to be positive from +/− till 4+ . If the sputum sample was not sufficient a new sample was taken.

**Statistical analysis**

Data analysis was performed using SPSS software version 11.0. Results are reported as median [25–75 percentiles]. Comparisons between groups were carried out using the non-parametric Mann-Whitney test. Statistical evaluation of the patient group before and during treatment was done with the
non-parametric Friedman test. A *p*-value < 0.05 was considered significant.

**Results**

From the 14 sputum cultures five patients had a positive culture. Three patients had isolated *Haemophilus influenza* and two patients had a culture with *Streptococcus pneumoniae* as well as *Haemophilus influenza*, respectively, *Moraxella catarrhalis* as well as *Haemophilus influenza*. However, none of these patients had evidence of an infection at admission, the X-ray showed no signs of an infection, the C-reactive protein and white blood cell count were in the normal range. Neither received antibiotics during the observation period despite six positive sputum cultures in retrospect.

At admission high levels of exhaled H₂O₂ were found in all patients. The median values before treatment were significantly higher compared with the two control groups, smokers and non-smokers (*P* < 0.001 for both control groups versus patient group). During treatment the median concentrations declined significantly (*P* = 0.001) (Fig. 1A, Table 1). There was also a significance between both control groups (*P* < 0.001 for smokers versus non-smokers; Table 2; Fig. 2).

Serum IL-8 before treatment was significantly elevated in comparison with the control group* (*P* < 0.001). The median values decreased significantly during treatment (*P* = 0.002) (Fig. 1B, Table 1).

sICAM-1 in serum before treatment was higher compared with the control group but differed not significant (*P* = 0.174). During treatment there was a significant decline of median values (*P* < 0.001) (Fig. 1C, Table 1).

sE-selectin in serum before treatment in comparison with the control groups was significantly higher (*P* = 0.015), but there was no significant decline of median values during treatment (*P* = 0.132) (Fig. 1D, Table 1).

The FEV₁ as well as the IVC did not change significantly during treatment (*P* = 0.085, respectively 0.135; Table 1) despite the significant difference with the control group* (*P* < 0.001, respectively 0.001).

*There was no significant difference between smokers and non-smokers (Table 2).

*G(see footnote *).

**Figure 1** Inflammation and oxidative stress in patients with exacerbated COPD. Measurements of exhaled H₂O₂ (A), IL-8 (C), sICAM-1 (D) and sE-selectin (E) were performed at day 1, 3, and 7.
Over time, patient 14 showed a gradual increase of the H$_2$O$_2$ concentration in breath condensate as well as E-selectin in serum. The concentration of ICAM-1 was significant increased (P<0.001) compared to the concentrations of ICAM-1 of the other patients at the three consecutive measurements.
Discussion

The H$_2$O$_2$ concentration in breath condensate as well as IL-8, E-selectin in serum of the patients at admission were significantly increased, while the spirometric lung tests FEV$_1$ and IVC where significantly decreased compared to the healthy controls (in both smokers and non-smokers). During treatment H$_2$O$_2$ in breath condensate and IL-8, E-selectin in serum decreased significantly.

The method applied to measure H$_2$O$_2$ concentration was valid and reproducible (see study design); therefore the observed differences between the consecutive measurements had to originate from biological mechanisms.

Hydrogen peroxide concentration can be influenced by the intake of food, beverages, smoking, exercise and an increase over the day. Most of these problems can be avoided by taking samples before the patient had breakfast at a fixed time point and a mouthwash with chlorohexidine 0.2% (see study design).

Dekhuijzen and co-workers reported comparable amounts of H$_2$O$_2$ in the expired air of unstable COPD patients. During treatment we observed a highly significant decline. At day 7 the H$_2$O$_2$ concentration in the treated patients resembled that of stable COPD patients.

IL-8 in serum is associated with a number of inflammatory pulmonary diseases in which neutrophils are implicated in the pathogenesis of the disease such as COPD. Increased levels of IL-8 in sputum as well as in plasma have been found in patients with COPD. This study showed significantly raised levels of IL-8 in serum of patients with exacerbated COPD before treatment compared to the control groups. Treatment resulted in a significant decrease of IL-8 during the observation period however a few patients had levels higher than normal after 7 days despite treatment. A fair consumption is that after stopping the prednisolone iv administration, the inflammatory process casu quo oxidative stress flared up again despite oral prednisolone. It is possible that the underlying inflammation was not completely resolved or a new inflammation started up during hospital stay. However, there is evidence that IL-8 production even occurs in the absence of an acute infection and may play a significant role in neutrophil accumulation in the chronic disease process.

An increased expression of the cell adhesion molecules ICAM-1 and E-selectin in the airways of patients with COPD has been found. ICAM-1 and E-selectin in serum are associated with inflammatory pulmonary reactions. These adhesion molecules are involved with leukocyte migration and accumulation at the sight of inflammation. There is an increased expression of both adhesion molecules in case of an inflammatory process. With X-ray, occasionally it may be possibly to detect this infiltration, however in this study the X-ray showed no signs of infiltration. Riise et al. detected soluble cell adhesion molecules ICAM-1 in serum and E-selectin in bronchoalveolar lavage fluid of patients with stable COPD. We only found significantly elevated levels of E-selectin in serum in case of exacerbated COPD in comparison with the control groups. There were still a few patients at day 7 who had higher E-selectin levels than the reference values. Although there was a tendency to decline during treatment, this was not significant. sICAM-1 in serum of the patients measured at admission proved to be significantly raised compared to the control groups. During treatment a significant decrease in time was found.

Spirometric evaluation showed no significant changes in IVC and FEV$_1$ after treatment had been started, however both spirometric parameters gave an improvement of the lung function (Table 1) during treatment with prednisolone.

In conclusion, our study clearly demonstrates enhanced oxidative stress as well as inflammation in the airways of the patients, admitted at the hospital because of exacerbated COPD. These results suggest that higher levels of sICAM-1 in serum and H$_2$O$_2$ in exhaled breath condensate may reflect the upregulation of ICAM-1 and H$_2$O$_2$ production due to inflammation. Therefore, we presume that sICAM-1 in serum and H$_2$O$_2$ in exhaled breath condensate are useful markers for disease monitoring in case of exacerbated COPD.

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