The interrelationship between markers of inflammation and oxidative stress in chronic obstructive pulmonary disease: modulation by inhaled steroids and antioxidant

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Summary Background: Chronic obstructive pulmonary disease (COPD) is accompanied by both airway and systemic inflammation and by oxidative stress. This study aimed to characterise the relationship between oxidative stress and inflammatory components in induced sputum and blood.

Material & methods: We studied blood and sputum samples from stable COPD patients (mean FEV\textsubscript{1} 60.5 \pm 7.5% predicted) at baseline (no treatment) and after 10 weeks treatment with either inhaled steroid, fluticasone propionate (FP) (1000 mg/d) or 10 weeks treatment with N-acetylcysteine (600 mg/d) (NAC). We assessed the inflammatory markers (IL-8, ECP, sICAM-1, NE) in sputum and serum and we compared them with blood markers of oxidative stress (SOD, GPx, TEAC, albumin, vitamin E and A).

Results: At baseline blood sICAM-1 correlated with IL-8 levels ($P$<0.01, $r=0.62$) and negatively with GPx ($P$<0.01, $r=-0.63$) and with TEAC ($P$<0.05, $r=-0.53$). TEAC correlated positively with GPx ($P$<0.01, $r=0.70$).

\textbf{KEYWORDS}

Chronic obstructive pulmonary disease; Oxidative stress; Inflammation

Abbreviation: COPD, chronic obstructive pulmonary disease; ECP, eosinophil cationic protein; FEV\textsubscript{1}, forced expiratory volume in 1 s; FVC, forced vital capacity; FP, fluticasone propionate; GPx, glutathione peroxidase; SICAM-1, soluble intercellular adhesion molecule-1; ICS, inhaled corticosteroids; IL, interleukin; NAC, N-acetylcysteine; NF-\kappaB, nuclear transcription factor-kappa B; ROS, reactive oxygen species; SOD, superoxide dismutase; TEAC, trolox equivalent antioxidant capacity

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Correlation between sICAM and IL-8 disappeared after NAC treatment. The correlation between sICAM and GPx disappeared after FP treatment. The correlation between TEAC and GPx was maintained after both NAC and FP.

Conclusions: The relationship between markers of inflammation, adhesion and antioxidant capacity is significantly modulated by treatment with N-acetylcysteine or inhaled corticosteroids.

Introduction

The pathogenesis of chronic obstructive pulmonary disease (COPD) is complex. It involves both airway inflammation\(^1\) and an oxidant/antioxidant imbalance.\(^2\) The inflammatory response is usually accompanied by an influx of neutrophils and release of proteases (neutrophil elastase, tryptase) and reactive oxygen species. Excessive transmigration is responsible for the enhanced number of neutrophils that is found in the airways and in a bronchoalveolar lavage fluid (BAL) of COPD patients.\(^3\) During this transmigration process various mediators are released. These involve cytokines that regulate the expression of adhesion molecules, inflammatory cell degranulation and migration.\(^4\) This inflammatory process is also accompanied by oxidative stress. Proinflammatory cytokines and growth factors stimulate release of reactive oxygen species (ROS) which act as signalling mediators for a variety of signal transduction pathways and gene expression.\(^5\)\(^-\)\(^7\) The transcription factors that have been implicated in many inflammatory responses are the nuclear factor-kappa B (NF-\(\kappa\)B) and activator protein-1 (AP-1).\(^8\)\(^-\)\(^9\) Both NF-\(\kappa\)B and AP-1 are sensitive to many different oxidative stress stimuli and some findings suggest that they may mediate cytokine and adhesion molecules expression.\(^10\)\(^,\)\(^11\) Moreover, NF-\(\kappa\)B is reported to be inhibited by antioxidants like \(N\)-acetylcysteine (NAC)\(^5\)\(^,\)\(^12\) and it could be also influenced by steroids.\(^13\) These observations suggest that inflammation and oxidative stress are co-dependent and strongly interrelated processes.

In order to characterise this interrelationship in COPD we assessed the correlation between the antioxidant defence markers (superoxide dismutase (SOD), glutathione peroxidase (GPx), trolox equivalent antioxidant capacity (TEAC), vitamin E and A), inflammation markers (intercellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), eosinophil cationic protein (ECP) and neutrophil elastase (NE). We also aimed to study the changes in the relationships after treatment with NAC or fluticasone propionate (FP) by analysing these interrelationships at baseline and after a 10-week period of treatment. This duration of treatment is based on studies which have demonstrated that treatments of at least 8 weeks inhaled steroids are required before the in vivo and anti-inflammatory effects become manifest.\(^14\)\(^,\)\(^15\)

Materials and methods

Twenty patients (16 male, 4 female) were recruited at The National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland. Inclusion criteria were: smoker or ex-smoker, not fully reversible airway obstruction with \(50\% < \text{FEV}_1 < 80\%\) and chest X-ray compatible with COPD. Patients with any history of atopy, asthma, renal and/or hepatic failure, major cardiac disease, cystic fibrosis, as well as patients who suffered an exacerbation of COPD in the last three months or used non-steroid anti-inflammatory medication, theophylline, long-acting beta stimulants or oxygen therapy were excluded from the study.

Study design

The study consisted of 4 periods of 10 weeks each and involved five visits. After a first 10-week period of wash-out, patients were randomised in a double blind manner to two groups. The first group \((n = 10)\) received oral NAC (Lysomucil\(^R\), Zambon, Poland) (600 mg, once a day) in combination with placebo for FP for 10 weeks. The second group \((n = 10)\) received placebo for NAC with inhaled FP via dry powder inhaler (DPI) (GSK, Poland) (1000 \(\mu\)g in two daily dosages of 500 \(\mu\)g). This was followed by a second wash-out period of 10 weeks; where after there was a cross-over to the alternative regime. Only short-acting beta agonists and/or anticholinergics (salbutamol or fenoterol and/or ipratropium bromide) were allowed during the whole period of the study. Patients were withdrawn from the study if they were treated with anti-inflammatory agents and/or oral steroids and/or theophylline. In case of acute exacerbation of COPD (AE) requiring treatment with an antibiotic, the observation period was prolonged for further two weeks after stopping with the antibiotic.\(^16\)
At each scheduled visit, induced sputum and fasting blood samples were collected and spirometry was performed to assess clinical stability.

**Spirometry and sputum induction**

FEV$_1$ measurements were performed before and 10 min after inhalation of 200 mg salbutamol. After spirometry, subjects inhaled 4.5% hypertonic saline aerosol, generated by a nebuliser (De Vilbiss, Somerset, PA, USA) during three periods of 5 min each. Following each period of hypertonic inhalation, subjects were asked to rinse their mouth and encouraged to cough and expectorate sputum into a sterile plastic container which was kept on ice. The procedure was stopped after three periods of 5 min or after a fall in FEV$_1$ 20% or more from the baseline.

**Sputum processing**

Sputum was processed within 2 h of collection. The weight of sputum plugs was determined and Sputolyisin (Calbiochem, CA, USA) (diluted 1:10 in PBS (Dulbecco’s Phosphate Buffered Saline) (Invitrogen, Paisley, UK)) added in a volume (µl) equal to 4 times the selected sample weight (mg). The samples were then mixed for 30 s on a mixer and further broken down by aspirating and dispensing several times with a disposable pipette. Then they were placed on a bench tube rocker for 15 min at 22°C. The samples were then filtered through a 48 µm nylon mesh (Cell Streiner, Becton Dickinson, Franklin Lakes, NJ, USA) in to a pre-weighted conical tube. Total cell count and viability were checked by trypan blue exclusion. The filtered samples were centrifuged at 500 g at 22°C for 10 min. The supernatant was aspirated and stored at −80°C. The pellet was resuspended in PBS to a concentration of 1 × 10$^6$ cells/ml and four cytospin slides were stained according to May-Grunwald-Giemsa (MGG). Differential cell count was performed by counting 400 cells in a blind way.

**Blood samples** were collected into sterile tubes containing either lithium-heparin or a clotting activator gel (S-Monovette System, 7.5 ml, Sarstedt, Numbrecht, Germany).

**Trolox equivalent antioxidant capacity (TEAC)** was measured spectrophotometrically in plasma at the wavelength 734 nm using the method of Re et al. TEAC value was calculated by means of the Trolox standard curve (% decolorisation vs. Trolox concentration).

**Glutathione peroxidase (GPx)** in full blood was measured with a Ransel Glutathione Peroxidase kit (Randox Laboratories Ltd., Antrim, UK) and is based on the method of Paglia and Valentine.

**Superoxide dismutase** in red blood cells was measured with RanSOD superoxide dismutase kit (Randox Laboratories Ltd., Antrim, UK).

**Albumin in sputum and serum** was measured according to the method of Doumas et al.

**Alpha-tocopherol and retinol** in serum were measured by High Performance Liquid Chromatography (Dionex, HPLC with a 100% methanol mobile phase) with detection at 292 and 325 nm, respectively, and a coefficient of variance (CV) of 10% and 13%, respectively.

**Eosinophil cationic protein (ECP)** was measured in serum and sputum using a fluoroenzymeimmunoassay provided by Pharmacia & Upjohn (Uppsala, Sweden). The assay is performed on UniCAP 100 instrument.

**Tryptase** was measured in sputum and serum applying the immunoassay from Pharmacia & Upjohn, according to the same principle as described for ECP.

**Neutrophil elastase (NE)** activity present in sputum and serum samples was measured spectrophotometrically using the synthetic substrate methoxysuccinyl-ala-ala-pro-val-paranitroanilide (MeOSAAPVpNa) (Sigma Chemicals, Bornem, Belgium). Aliquots of 50 µl of standard or sample were added to wells of a microtiter plate (Life Technologies, Ltd., Paisley, UK) followed by MeOSAAPVpNa solution. The reaction was continued for 20 h at 37°C. The absorbance was read at 405 nm.

**Soluble ICAM-1** in sputum and serum was measured with ELISA: test plate Human sICAM-1, Biosource International, Camarillo, USA. The valid analytical range is 0.625–10 ng/ml.

**Interleukin-8 and 10** in both sputum and serum were measured with Flexia™, Biosource International, Camarillo, USA. Valid analytical range of IL-8 is 3.6–750 pg/ml and of IL-10 is 6–1000 pg/ml.

**Statistical analysis**

The Pearson correlation coefficient was used to test for correlation between parameters. Statistical significance was assumed when the two-tailed $P$ value was less than 0.05. Data were analysed using Statistica 5.1, StatSoft, Inc., Tulsa, OK, USA.

**Results**

The results present an analysis of 20 patients who provided adequate samples at each visit. Two patients dropped out after the fourth visit (the
second wash-out period): one because of protocol violation and the other one due to prolonged exacerbation of COPD. Three patients (included in the analysis) experienced AE of COPD (one of them during FP, two during NAC treatment). The subject characteristics are given in Table 1. Parameters were not significantly different after each wash-out period. All patients were studied in the stable clinical state.

**Lung function at baseline**

At baseline, there was no correlation between lung function (FEV1% predicted) and any of the inflammation and oxidative stress markers.

**Correlation between markers of inflammation**

At baseline was a positive correlation between serum sICAM-1 and serum IL-8 levels ($P<0.01$, $r=0.62$). This correlation was maintained after FP ($P<0.01$, $r=0.65$) but disappeared after NAC treatment ($P=0.38$, $r=0.22$) (Figs. 1A–C). There was no correlation between NE and the other markers. Moreover, there was no correlation between neutrophil numbers and any of the markers in the induced sputum. The sputum markers were measurable within the accepted ranges of the kits (for example, ICAM-1 in sputum values ranged from 0.68 to 10 ng/ml, IL-8 from 23 to 3000 pg/ml and ECP from 2 to 1350 ng/ml).

**Correlation between inflammatory and oxidative stress markers**

At baseline serum sICAM-1 correlated negatively with GPx ($P<0.01$, $r=-0.63$). This was maintained after NAC treatment period ($P<0.01$, $r=-0.67$) but not after FP ($P=0.15$, $r=-0.35$) (Fig. 2A–C). Serum sICAM-1 also correlated negatively with TEAC at baseline ($P<0.05$, $r=-0.57$) as well as after FP ($P<0.05$, $r=-0.50$) and NAC ($P<0.01$, $r=-0.69$) treatment periods.

**Table 1** Patient's characteristics at visit 0.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients ($n$)</td>
<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.6 ± 2.1</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>16/4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.6 ± 1.1</td>
</tr>
<tr>
<td>Current smokers/ex-smokers</td>
<td>11/9</td>
</tr>
<tr>
<td>Smoking (packyears)</td>
<td>36.7 ± 3.7</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>60.5 ± 1.7</td>
</tr>
<tr>
<td>FEV1 (% predicted) post-salb</td>
<td>65.1 ± 2.6</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>84.1 ± 2.6</td>
</tr>
<tr>
<td>FVC (% predicted) post-salb</td>
<td>87.9 ± 2.8</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>54.1 ± 2.3</td>
</tr>
<tr>
<td>FEV1/FVC post-salb</td>
<td>55.2 ± 2.6</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM, $n$=number of patients, FEV1: forced expiratory volume in 1 s, FVC: forced vital capacity, post salb: after salbutamol administration (200 μg).
Moreover, serum IL-8 levels correlated negatively with GPx ($P < 0.05$, $r = -0.45$) and TEAC ($P < 0.05$, $r = -0.52$) at baseline. Serum IL-8 correlated negatively with GPx and TEAC after FP ($P < 0.05$, $r = -0.47$ and $P < 0.05$, $r = -0.50$, respectively) but not after NAC treatment (Figs. 3 and 4A-C).

**Correlation between oxidative stress markers**

TEAC correlated positively with GPx during all periods: at baseline ($P < 0.01$, $r = 0.7$), after FP ($P < 0.001$, $r = 0.77$) and after NAC ($P < 0.05$, $r = 0.52$) (Figs. 5A–C).

There are no correlations between SOD, albumin, retinol and tocoferol and the inflammatory markers (data not shown).

**Discussion**

This study analyses the correlations between inflammatory and oxidative stress markers in stable COPD patients receiving no anti-inflammatory
treatment or after treatment with an anti-oxidant (NAC) or an inhaled corticosteroid (FP). Previous studies have shown that ROS released from circulating neutrophils is associated with the development of the airflow limitation in COPD patients. The pathogenic mechanisms explaining this association are multifactorial and involve an intricate interplay between inflammatory and oxidative processes. On the one hand, these ROS, by activating NF-κB and other redox-sensitive transcription factors such as AP-1, cause an increased gene expression of both pro-inflammatory cytokines and of protective enzymes. On the other hand, these cytokines play an important role as activators of neutrophils and as chemoattractants, which will, in their turn, determine the mediators released from neutrophils. The correlation we observed between ICAM-1 and IL-8 at baseline supports the idea that inflammation, which is reflected by increased IL-8 levels, promotes the expression of adhesion molecules (ICAM-1).24
However, the interrelationship between neutrophils and chemoattractants is more complex because IL-8 is released not only from neutrophils but also from epithelial cells. Moreover, there is also another important mediator leukotriene B4 (LTB4) that participates in the neutrophil response. The lack of correlation between NE and IL-8 observed in our study suggests that there are multiple sources of IL-8 and that additional factors play a role in NE and IL-8 release.

The relationship between inflammation and oxidative stress indices is also complex. The common factor for both pathways is NF-κB, which, when activated, induces the expression of cytokines and adhesion molecules in a positive feedback loop. Moreover, this pathway is also redox-dependent and is activated by oxidative stress. Therefore, it can be postulated that antioxidant defence capacity may affect inflammatory response. For example, increased antioxidants in blood could, by inhibiting NF-κB, lead to a decrease in both IL-8 and ICAM-1 levels. In this study we assessed antioxidant defence by monitoring GPx, as example of an antioxidant enzyme and TEAC, as example of the non-enzymatic antioxidant capacity of plasma. The strong positive correlation between TEAC and GPx at all points during the study supports the fact that these two antioxidant mechanisms are tightly related. Moreover, GPx and TEAC correlated negatively with blood levels of sICAM-1 and IL-8 at baseline. These results support our hypothesis of a negative feedback loop between antioxidant capacity and inflammatory response.

NAC, a thiol-containing compound, is known for its antioxidant activity. By acting as a direct scavenger of ROS it protects cells against damage by H2O2. As a source of sulfhydryl groups in cells and by increasing reduced glutathione levels in cells, it decreases NF-κB activity, and neutrophil hyperreactivity.

In our group of patients treatment with the antioxidant NAC tended to decrease IL-8 levels but did not affect TEAC, GPx or ICAM-1 (manuscript in preparation). The change in IL-8 levels was accompanied by a disappearance of the negative correlation between this pro-inflammatory cytokine and GPx and TEAC. This suggests that NAC affected IL-8 expression by pathways not involving GPx and plasma antioxidants. The disappearance of the positive correlation between IL-8 and ICAM-1 after NAC supports the concept that ICAM-1 expression is additionally or even alternatively modulated by pathways which are not dependent on NF-κB activity and which are therefore not as susceptible to the effects of NAC treatment as, for example, IL-8. For example PKC, the IFN-γ dependent JAK/STAT pathway and MAP kinases have all been shown to act in concert and to co-activate ICAM-1 expression depending on the cell type and on the type of stimulus. Some of these pathways (MAPK and protein PKC) can be induced by IL-8 in human neutrophils, but others are IL-8-independent. IL-8, in its turn, is regulated not only by activation of the NF-κB transcription factor but also through other pathways such as protein kinase A (PKA), protein kinase C (PKC) and p38 mitogen activated protein kinase (MAPK). This phenomenon of multiple regulatory mechanisms can explain the unequal down-regulation of IL-8 and ICAM-1 that we observed after treatment with NAC. A similar observation in sepsis patients was attributed to the differences in the kinetics of the cytokine and adhesion molecule since it takes longer for soluble ICAM-1, which is shed by the endothelial cells, to reach the blood circulation. And lastly, it is plausible that the changes in redox status (GSH/GSSG ratio) needed to affect NF-κB-dependent transcription might be higher for ICAM-1 than for IL-8, a phenomenon observed for E-selectin and VCAM.

In contrast to NAC, FP treatment which led to a significant increase in antioxidant capacity (GPx and TEAC) was accompanied by a loss of the negative correlation of GPx with ICAM-1. FP did not affect IL-8 or ICAM-1 levels and consequently had no effect on the correlations between this cytokine and ICAM-1 or antioxidants. This lack of effect is in concordance with the observation that FP might act through NF-κB-independent pathways. Recent literature shows that GPx is regulated by AP-1.

In summary, we assessed some of the relationships between inflammatory markers and oxidative stress markers and pointed out to significant modulation of these interrelationships by treatment with FP and NAC. Understanding the relationship between these processes requires more studies with specific inducers and inhibitors directed against intracellular mediating pathways such as p38 MAP kinase and NF-κB as well as antisense oligonucleotides. Elucidating the processes behind the inflammatory response in COPD and their relation to oxidative stress may potentially contribute to the development of preventive and therapeutic strategies that will slow down the process of lung destruction.

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Reference

