8-isoprostane as a marker of oxidative stress in non-symptomatic cigarette smokers and COPD

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Short title: 8-isoprostane and cigarette smoking
ABSTRACT

Study objectives: 8-Isoprostane is a potential in vivo marker for oxidant burden, but its usefulness in induced sputum of smokers and COPD has not been investigated.

Design: We investigated 58 subjects, i.e. 11 never smokers, 11 ex-smokers, 13 healthy current smokers, 23 COPD patients with Stage 0-III disease (GOLD criteria). 8-Isoprostane was determined from induced sputum by enzyme immunoassay.

Results: Sputum 8-isoprostane levels were similar in the never-smokers and ex-smokers, but were elevated in the healthy smokers compared to non-smokers (p=0.005) and in Stage I-III COPD (p<0.0001 vs non-smokers and p=0.02 vs healthy smokers). Sputum 8-isoprostane levels could not differentiate non-symptomatic smokers from those with Stage 0 COPD. There was a correlation between the sputum 8-isoprostane level and lung function parameters (FEV1/FVC r=-0.66, p<0.0001) and sputum neutrophils (r=0.37, p=0.02).

Conclusions: The sputum 8-isoprostane level correlates with the severity of COPD. However, it does not appear to differentiate healthy smokers from those who are at risk for developing COPD (GOLD Stage 0).

Word count: 162

Keywords: Cigarette smoking, COPD, oxidant, sputum
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is generally diagnosed when lung function parameters have become significantly reduced and a major part of the lung has been damaged. The pathogenesis of COPD has been strongly associated with reactive oxygen species (ROS) [1-4] although it is not known how oxidative/nitrosative stress predicts the disease progression. Several oxidant markers and “footprints” of oxidative/nitrosative damage have been detected in COPD lung tissue, sputum, exhaled air and exhaled breath condensate, [3-6] but it has not been resolved unequivocally whether these bio-markers can be used in the early assessment of cigarette smoke related lung diseases, their progression or do they relate to smoking alone.

8-epi-PGF$_{2\alpha}$ (8-isoprostan e) has been suggested to be the most reliable approach to monitor oxidative stress in vivo [7,8]. Isoprostanes are formed by free-radical-catalyzed lipid peroxidation of arachidonic acid and in cell membrane phospholipids. Isoprostanes can also be released into the circulation, secretions and urine where they have been found to be stable and reproducible in many experimental approaches [8,9]. Isoprostanes also have potent biological actions and therefore they may significantly contribute to the progression of oxidant mediated lung diseases, such as COPD.

Several studies have shown elevated 8-isoprostan e in the exhaled breath condensate of COPD patients [10-13]. There are, however, a number of uncertainties with respect to the usefulness and standardization of the exhaled breath condensate [14,15-17]. One recent study also failed to detect 8-isoprostan e in the exhaled breath condensate in the majority of cigarette smokers [18], the reasons being suggested were the high dilution of all biological constituents in the breath condensate and the low sensitivity of the 8-isoprostan e enzyme immunoassay (EIA) method. Induced sputum is a
standardized method that reflects local airway inflammation reliably. 8-Isoprostane has not been previously investigated from the induced sputum of smokers.

The present study was undertaken to obtain a more accurate insight into the significance of the local oxidant burden in the airways of healthy smokers and smokers who evidently have a risk for COPD (Stage 0 COPD, GOLD criteria) (the Global Initiative for Chronic Obstructive Lung Disease) [19] by analyzing the concentrations of 8-isoprostane from induced sputum specimens. This study included age matched subjects, never smokers, ex-smokers, “healthy smokers”, smokers with Stage 0 COPD (symptoms with normal lung function parameters), GOLD criteria [19], and with stable COPD (Stage I-III COPD).

**Subjects and methods**

**Subjects**

A total of 58 subjects, 11 never smokers, 11 ex-smokers (who had stopped smoking at least 20 years before the study with a smoking history of less than 15 packyears), 13 healthy non-symptomatic smokers and 9 smokers with symptoms (S Georges questionnaire for symptoms such as cough and sputum production) whose lung function parameters were normal (Stage 0 COPD) [19], and 14 COPD patients with Stage I-III disease were included. The diagnosis of COPD was based on the GOLD criteria with postbronchodilator forced expiratory flow in one second (FEV1)/forced vital capacity (FVC) <70% with the post-bronchodilator effect <12%. Atopy and allergies were excluded. Healthy controls and Stage 0 COPD had not been treated with any anti-inflammatory medication for 2 months, but two individuals had been prescribed with a short acting bronchodilator. The medications of the Stage I-III included inhaled short acting bronchodilators in 100% of cases, long acting bronchodilators in 79% and inhaled steroids in 64%. None of the
subjects were allowed to smoke during the 12 hours before collection of the specimens and none had suffered from any viral infection for 2 months. Each subject underwent spirometry with the bronchodilator test and an assessment of total lung capacity and diffusion capacity [20].

The characteristics of the subjects are shown in Table I.

This study was approved by the Ethics Committee of Helsinki University Hospital with written consent was obtained from every subject. The study is registered by the hospital (www.hus.fi/clinicaltrials).

Sputum processing

Sputum was induced as described by the ERS task force with 4.5% physiologic saline solution [21]. Sputum was processed as previously described [22]. Briefly, expectorated samples were processed with four volumes of dithioerythritol (DTE, Sigma, Germany). Suspensions were filtered through 70-µm nylon gauze and centrifuged at 400 g at 4°C for 10 min. In preliminary studies the sputum was also collected by the same protocol in phosphate buffered saline (PBS) without DTE to test the possible effect of DTE on the 8-isoprostane EIA analysis (see below). After centrifugation, the pellet was resuspended, and the viabilities and absolute numbers of cells were calculated by the trypan blue exclusion test. All samples in DTE or PBS were immediately frozen at -80°C. Cytospins were prepared and stained using May-Grunwald-Giemsa (MGG) method for cell differential counts. The cytospins were frozen at –20°C.

Analyses

Free 8-isoprostane was analysed by EIA according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI). The values were expressed as pg/ml. The EIA method is highly specific for 8-isoprostane and has been earlier used for the assessment of 8-isoprostane from
exhaled breath condensate [10,23], bronchoalveolar lavage [24], plasma [25] and induced sputum [26]. The method has been found to show strong association with the results obtained with gas chromatography-mass spectroscopy (GC-MS) [26]. To test the analysis further, sputum samples were analyzed at three different dilutions; these results gave good reproducibility (%CV=10.5, ICC= 0.87 ). When one individual sputum specimen divided originally in small aliquots was run in ten separate assays the reproducibility was good; the values of this specimen varied between 29.7-37.1 pg/ml, SD 3.8. The effect of DTE had been tested in the preliminary studies by adding the corresponding DTE concentration as in the sputum specimens to the standards, these determinations showed very consistent results (r=0.99). In addition, eight inductions had been processed with PBS without DTE. These specimens were further divided to two parts and treated with DTE or PBS, and analyzed for 8-isoprostane. Also these results with or without DTE were very similar (r=0.75). The dilutions were made in the buffer provided by the manufacturer.

Statistics

All the statistical analyses were performed using the SPSS 10.0 software program (SPSS Inc., Chicago, IL, US). Data is shown as mean ± standard error of the mean (SEM) or median and range for not normally distributed data. Data for all groups was analysed by the Kruskall-Wallis test and differences between individual variables from two groups were analysed by the Mann-Whitney U-test. Correlations between variables were sought using the Spearman rank correlation test. A p-value of <0.05 was considered significant. For values below the detection limit in 8-isoprostane analysis, we used random number interpolation. This was needed for only five samples in the healthy control group.
RESULTS

As expected, current smokers had higher numbers of neutrophils in the induced sputum than non-smokers (Table 1).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Healthy non-smokers</th>
<th>Healthy smokers</th>
<th>COPD stage 0</th>
<th>COPD stage 1 or more</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>22</td>
<td>13</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Male/female</td>
<td>18/4</td>
<td>9/4</td>
<td>8/1</td>
<td>7/7</td>
</tr>
<tr>
<td>Age</td>
<td>58±1.9</td>
<td>53±1.6</td>
<td>63±1.6</td>
<td>58±1.9</td>
</tr>
<tr>
<td>BMI</td>
<td>26±0.8</td>
<td>27±1.3</td>
<td>28±0.8</td>
<td>27±0.8</td>
</tr>
<tr>
<td>Pack-years*</td>
<td>7±3.0</td>
<td>30±3.8</td>
<td>54±5.9</td>
<td>44±3.0</td>
</tr>
<tr>
<td>Post-bronchodilator</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC (l)</td>
<td>4.6±0.24</td>
<td>4.7±0.42</td>
<td>3.4±0.14</td>
<td>2.9±0.10</td>
</tr>
<tr>
<td>FVC (% pred)†</td>
<td>100±3.8</td>
<td>95±5.0</td>
<td>82±4.5</td>
<td>80±2.8</td>
</tr>
<tr>
<td>FEV1 (l)*</td>
<td>3.7±0.15</td>
<td>3.8±0.33</td>
<td>2.6±0.12</td>
<td>1.7±0.10</td>
</tr>
<tr>
<td>FEV1 (% pred)*</td>
<td>100±3.9</td>
<td>96±5.0</td>
<td>77±4.1</td>
<td>57±2.8</td>
</tr>
<tr>
<td>FEV1/FVC*</td>
<td>80±1.4</td>
<td>80±1.3</td>
<td>76±1.1</td>
<td>57±1.4</td>
</tr>
<tr>
<td>DLCO (%)*</td>
<td>96±2.8</td>
<td>86±3.7</td>
<td>80±4.1</td>
<td>58±2.8</td>
</tr>
<tr>
<td>Sputum neutrophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)*</td>
<td>27 (0-75)</td>
<td>37 (0-93)</td>
<td>72 (32-84)</td>
<td>74 (46-82)</td>
</tr>
<tr>
<td>Sputum neutrophils</td>
<td>0.10</td>
<td>0.31</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>x 10⁶/g‡</td>
<td>(0-0.76)</td>
<td>(0-1.65)</td>
<td>(0.11-1.32)</td>
<td>(0.07-1.86)</td>
</tr>
<tr>
<td>Sputum macrophages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)*</td>
<td>41 (20-97)</td>
<td>51 (6.8-70)</td>
<td>26 (11-67)</td>
<td>26 (13-99)</td>
</tr>
<tr>
<td>Sputum macrophages</td>
<td>0.19</td>
<td>0.21</td>
<td>0.34</td>
<td>0.14</td>
</tr>
<tr>
<td>x 10⁶/g</td>
<td>(0.04-0.83)</td>
<td>(0.08-0.90)</td>
<td>(0.04-1.18)</td>
<td>(0.06-0.67)</td>
</tr>
</tbody>
</table>

Data is shown as mean±SEM or median (range), *p<0.0001, † p<0.05, ‡ p<0.01, (between all groups, Kruskall-Wallis test)

The percentage and number of sputum neutrophils were very similar in never smokers and ex-smokers (who had stopped smoking for at least 20 years ago). The percentage of sputum neutrophils tended to be higher in those smokers who had symptoms but nonetheless normal lung function parameters (Stage 0 COPD) compared to non-symptomatic smokers (p=0.06, Table 1). However, there was no difference in the total number of neutrophils between these two groups (p=0.23).
The levels of 8-isoprostane in the induced sputum were higher in healthy smokers (median 108.4 pg/ml) than in non-smokers (median 15.3 pg/ml) (p=0.005) but did not differ between healthy smokers and Stage 0 COPD (median 66.6 pg/ml) (Fig 1a). The levels of 8-isoprostane did not differ between never-smokers and ex-smokers. The levels significantly increased in COPD (median 202.2 pg/ml (Stage I-III) (p<0.0001 compared to non-smokers and p=0.02 compared to healthy smokers). There was a significant correlation between the 8-isoprostane levels and lung function parameters (8-isoprostane vs FEV1/FVC r=-0.66, p<0.0001, vs FEV1 r=-0.48, p=0.006) (Fig 1b) and between the 8-isoprostane level and smoking history evaluated from the pack years (r=0.56, p=0.001) (Fig 1c). The 8-isoprostane level in the sputum significantly correlated with sputum neutrophils (total neutrophils: r=0.37, p=0.02) (Fig 1d). Three subjects (33%) with stage 0 COPD had stopped smoking at least one year ago (mean time from quitting two years). However, there was no difference between these ex-smokers and current smokers in Stage 0 COPD group in any of the parameters measured. In patients with Stage I or worse COPD, six patients (43%) had stopped smoking (mean time from quitting 3.6 years). In this group, current smokers tended to have higher sputum 8-isoprostane levels than ex-smokers but the difference was not significant (p=0.09). No significant differences could be found between current smokers and ex-smokers in these COPD-patients. When all the subjects were divided into two groups, current smokers and non-smokers, then the level of 8-isoprostane in sputum was significantly higher in current smokers (p=0.001). In the COPD group, 64% of the patients used inhaled steroids. However, there were no significant differences between 8-isoprostan levels in patients with inhaled steroids or without them.

**DISCUSSION**

The major interest of the present study was to assess 8-isoprostane in induced sputum specimens since these specimens are probably the most sensitive non-invasive way of assessing oxidative
stress in the airways. As far as we are aware there are no studies on the levels of 8-isoprostane in
the induced sputum of COPD. The levels of 8-isoprostane were already significantly elevated in
healthy smokers and consistently increased in COPD and we noted a significant correlation with the
lung function parameters.

The EIA method used appeared to be reproducible. Several earlier studies have also assessed 8-
isoprostane in the exhaled breath condensate and bronchoalveolar lavage [10,23,24]. Our results are
also in line with a recent study where this same method was used to examine the induced sputum of
asthma and found to have highly significant correlation (r>0.9) with gas chromatography-mass
spectroscopy (GC-MS) and 100% spiking recovery [26].

Given that 8-isoprostane was also related to smoking without COPD, we investigated if sputum 8-
isoprostane levels can differentiate healthy smokers from those who are at risk of developing
COPD. Therefore we included healthy smokers and those who were exhibiting symptoms i.e. Stage
0 COPD. The levels of 8-isoprostane were significantly elevated in smokers but there was no
difference in the sputum 8-isoprostane level between non-symptomatic smokers and Stage 0 COPD.
None of the smokers had smoked for 12 hours. 8-isoprostane was, however, significantly higher in
the sputum of COPD (Stage 1 or more) when compared to the Stage 0. These results clearly suggest
that 8-isoprostane is not a reliable marker in differentiating healthy smokers from those who
probably have a risk of suffering COPD. It needs to be emphasized also that Stage 0 COPD does
not necessarily lead to the development of COPD [27-30]. Overall, 8-isoprostane is already elevated
in the sputum of smokers but it still remains unclear whether this or other markers of oxidative
stress are sensitive enough in finding those smokers who really are at risk for COPD development.
In conclusion, 8-isoprostane levels are clearly increased in the induced sputum of smokers and especially in moderate-severe COPD but do not appear to be very sensitive in differentiating healthy smokers from those who probably have a risk of developing COPD.

Acknowledgements

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The authors declare that they have no competing interests.
Reference List


FIGURE LEGENDS

Figure 1

8-isoprostane levels (pg/ml) in sputum (1a) samples. Median values are shown with horizontal bars. P-values between all groups were calculated with Kruskall-Wallis test and between two groups with Mann-Whitey test (only significant p-values shown). Correlations (Spearman rank correlation test) between FEV1/FVC and sputum 8-isoprostone (1b), between pack-years and sputum 8-isoprostane (1c), and between total numbers of sputum neutrophils and sputum 8-isoprostone (1d).