MARKERS OF PULMONARY DISEASES IN EXHALED BREATH CONDENSATE

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Abstract. Exhaled breath condensate has been more and more extensively used as a novel and non-invasive method to study airway inflammation. It is simple to perform, very well tolerated by patients and no adverse events have been reported so far. Serial measurements can be made with no harmful effects on patients, which is of extreme value in occupational medicine. Exhaled breath condensate has been obtained from both adult and children patients suffering from various pulmonary diseases such as asthma, cystic fibrosis, chronic obstructive pulmonary disease, and interstitial lung diseases. Several markers and mediators are detectable in breath condensate: hydrogen peroxide, thiobarbituric acid–reactive substances, isoprostanes, prostaglandins and leukotrienes. Nitric oxide–related markers have also been studied in the condensate. There is increasing body of evidence that changes in condensate markers reflect local abnormalities of airway lining fluid.

Key words:
Exhaled breath condensate, Hydrogen peroxide, Leukotrienes, Nitrotyrosine, Asthma, Chronic obstructive pulmonary disease

INTRODUCTION

Airway inflammation plays an important role in various respiratory lung diseases, including recurrent wheezing, asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [1,2]. Several attempts have been made therefore to detect and monitor inflammatory changes and mediators using non-invasive methods. There is also a need for objective and early criteria to evaluate airway inflammation. The first useful and valuable step has been made by measuring bronchial hyperresponsiveness to methacholine, which, indeed, provides complementary information on symptoms, lung function, and the course and prognosis of diseases. More recent efforts have been made to detect markers in induced sputum and exhaled markers of airway inflammation [3]. The diagnosis of occupational lung diseases in general and occupational asthma in particular, needs to be more objective and based on a wider range of criteria. Frequently used measurements (metacholine airway responsiveness, diurnal and at work variations of peak expiratory flow, cannot be performed optimally and are often insufficient in occupational medicine, and further measurements may prove more useful. Measurements of exhaled breath condensate (EBC) that can be made at workplace directly and noninvasively, and last but not least repeatedly over short periods of time could be valuable in occupational medicine. Inflammatory mediators in EBC that increase at work in most patients with occupational lung disease can be used as objective evidence to support the diagnosis.

Unfortunately, the collection of sputum induced by inhalation of hypertonic saline may irritate the airways and subsequently becomes similar to bronchial challenge tests [4]. Repetitive measurements are also not recom-
mended due to cell count changes and proinflammatory action of sputum induction over short period of time. Exhaled nitric oxide (NO) is the most extensively studied exhaled marker, and abnormalities in NO levels have been documented in several lung diseases [5].

Recently, EBC has been more and more extensively used as a novel and non-invasive method to study airway inflammation. It is extremely simple to perform, very well tolerated by patients and no adverse events have been reported so far. Moreover, it seems valuable in occupational medicine, in which serial measurements to monitor the impact of work environment and exposure estimates on lung function can be made without harmful effects on patients. The measurement can be performed in each subject and be repeated as often as possible. A 15-min time of collection seems to be sufficient to obtain samples. If analysis is restricted to a single marker or mediator only a few minutes of tidal breathing are sufficient for an adequate sample. Forced breathing should be avoided. Sampling can be interrupted any time. Condensate collection should not be performed on the same day or in case of respiratory insufficiency requiring support of respiration. The volatile and non-volatile substances in human breath can potentially be used in the assessment of airway inflammation, based on the assumption that aerosol particles and vapor in exhaled air reflect the composition of the lower airway fluids. However, there are no sufficiently convincing data providing clear evidence on the source of particles present in EBC, and only theoretical considerations have arrived at the conclusion that the exhaled breath and exhaled breath condensate mainly represent the biochemical situation in the lower airways.

Breath condensate mainly consists of water but also of inorganic salts and oxides of about 10 ppm, lipids, hydrocarbons and proteins. The reliable determination of these compounds is fundamental for diagnosing respiratory disorders using exhaled breath condensate (Table 1).

The composition of the condensate is very complex. There are several hints indicating that EBC consists of two phases (vapor and aerosol), which constitute breath condensate. The vapor phase consists mainly of non-water soluble biomolecules potent to form binary systems with water in the watery surface of the lung [6]. Leukotrienes and prostaglandins are good examples of molecules that normally are only hardly volatile and can evaporate easily in the vapor phase of breath condensate [7]. Many proteins can be expired via the aerosol phase of the condensate. As shown by Papineni [8] the lungs produce aerosols under tidal breathing conditions, and a relatively low number of particles per liter of exhaled air can be increased if a deep exhalation maneuver or coughing before the breath analysis is performed. These findings confirm a hypothesis that aerosol particles are formed in the peripheral compartments of the lung and are exhaled.

Condensate constituents reflect different molecules from oral cavity and oropharynx, alveoli, and their proportional contribution has not as yet been clearly defined.

Table 1. Markers of inflammation in exhaled breath condensate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Asthma</th>
<th>COPD</th>
<th>Smokers</th>
<th>CF</th>
<th>ARDS</th>
<th>Bronchiectases</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8-Isoprostane</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Thiobarbituric acid-reactive products</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Nitrosothiols</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Nitrite/Nitrates</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>pH</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
However, a general idea of breath condensate collection consists in the expiration of breathing air through a condenser cooled down by ice, dry ice or by cooling liquid (Fig. 1). An important factor for the sample quality seems to be temperature, the lower the better especially for eicosanoids that are highly unstable. There is also a risk of saliva contamination, which potentially influences the levels of different markers in breath condensate. Saliva is a reach source of hydrogen peroxide and high levels of eicosanoids have been observed in saliva from asthmatic patients. Therefore, amylase as a good marker of saliva contamination should be monitored when obtaining breath condensate and the subjects should be asked to rinse the mouth before and during collection.

In the airways, EBC provides an easy to perform means of exploring the airways without the need to undertake invasive procedures, such as bronchoscopy.

MARKERS OF OXIDATIVE STRESS

Hydrogen peroxide (H$_2$O$_2$)

An important source of H$_2$O$_2$ are phagocytes. Activated cells generate O$_2^-$, which is converted to H$_2$O$_2$ by superoxide dismutase and hydroxyl radical, formed non-enzymatically in the presence of Fe$^{2+}$. An intense airway inflammation can be caused either by H$_2$O$_2$ alone or by newly generated hydroxyl radical [10]. H$_2$O$_2$ is released in extracellular fluid. In the airways, part of H$_2$O$_2$, which has not been decomposed by antioxidant enzymes, can be exhaled with exhaled breath. H$_2$O$_2$ is elevated in EBC in various inflammatory lung disorders, such as asthma [11,12], CF [13], bronchiectasis [14], adult respiratory distress syndrome (ARDS) and acute hypoxemic respiratory failure [15], cigarette smoking [16], and COPD [17]. Cigarette smoking is a strong pro-inflammatory and pro-oxidant factor and therefore, high levels of H$_2$O$_2$ have been found in EBC from smokers compared to non-smoking subjects [18]. Increased H$_2$O$_2$ levels have been found in EBC in a relatively small group of asthmatic children, mainly in those with acute disease, however, elevated H$_2$O$_2$ in stable asthmatics has been also observed [19]. As postulated by Horvath and co-workers [20] measurements of H$_2$O$_2$ in EBC and exhaled NO in asthmatic patients provides complementary data for monitoring the disease progress. Exhaled H$_2$O$_2$ may be used not only as a diagnostic test but also to guide anti-inflammatory treatment. Inhaled beclomethasone in low dose has been shown to decrease H$_2$O$_2$ in EBC in a 2-week treatment [21]. This has been also observed in children with stable asthma, part of them received inhaled corticosteroids daily [12]. There was a significant difference in median H$_2$O$_2$ concentration between asthmatics without anti-inflammatory treatment and healthy controls. A study of ARDS patients treated with corticosteroids showed a tendency towards lowering the levels of H$_2$O$_2$ in the expired air condensate as compared to steroid-naive ARDS patients [22]. In a
recent study, it has been observed that long-term treatment of N-acetylcysteine (600 mg daily) decreases H₂O₂ exhalation in COPD patients [23]. Both, asthma and CF patients with an acute pulmonary exacerbation have abnormally high levels of exhaled H₂O₂, which decrease during antibiotic treatment [24].

**Products of lipid peroxidation**

**Thiobarbituric acid-reactive substances**

Measurement of thiobarbituric acid-reactive substances (TBARS) is a simple method, but non-specific to assess lipid peroxidation damage in tissues, cells and body fluids. TBARS levels are increased in exhaled breath condensate in asthma [11] and COPD [25] and they increase during exacerbations [24]. Increased levels of conjugated dienes in EBC in bronchial biopsies from patients with COPD and simple chronic bronchitis have also been observed [26].

**8-Isoprostane**

8-Isoprostane, stable prostaglandin-like arachidonate product formed by ROS on membrane phospholipids is postulated to be a reliable biomarker of lipid peroxidation caused by reactive oxygen species and represent a quantitative measure of oxidant stress *in vivo* [27]. 8-Isoprostane appears to reflect oxidative stress in EBC. It progressively increases with increasing severity of asthma, reaching very high levels in aspirin-induced asthma [28,29]. It is also reported to be increased in EBC in COPD patients [30] and further goes up in exacerbated COPD [31]. There was also observed a positive correlation between exhaled H₂O₂ and 8-isoprostane levels, which might reflect the cause-effect relationship (A. Antczak, unpublished data). 8-Isoprostane has also been detected in EBC from ARDS patients [32].

**Nitrogen-reactive species**

**Nitrotyrosine**

Nitrotyrosine formation in EBC may be a marker of nitrosative-oxidative stress in airways. NO is able to react with superoxide anions in the airways to form peroxynitrite, a highly reactive species. Peroxynitrite in reaction with tyrosine residues in proteins forms a stable product - nitrotyrosine. Nitrotyrosine concentrations have been detected in EBC of normal subjects, and were increased significantly in patients with mild asthma [33]. However, the levels of nitrotyrosine in EBC have been higher in mild than in moderate or severe asthma. Moreover, there was a significant correlation between nitrotyrosine in EBC and exhaled NO in patients with mild asthma [33]. Exhaled NO is decreased in CF patients, perhaps because it is metabolized to oxidative end-products. 3-Nitrotyrosine may indicate local ROS formation. Nitrotyrosine levels in EBC were significantly increased in stable CF patients compared with normal subjects. There was an inverse correlation between the levels of nitrotyrosine and the severity of lung disease [34]. Production of nitrotyrosine may reflect increased formation of reactive nitrogen species, such as peroxynitrite or direct nitration by granulocyte peroxidases, indicating increased oxidative stress in airways of CF patients [34].

**Nitrosothiols**

Nitrosothiols (RS-NOs) are formed by interaction between NO and glutathione. RS-NOs are detectable in EBC of healthy subjects and are increased in patients with inflammatory lung diseases. RS-NOs in EBC were higher in subjects with severe asthma versus normal subjects and those with mild asthma [35]. Elevated RS-NOs were also found in CF patients and in smokers. In current smokers, RS-NOs values correlated with smoking history (pack/year). As RS-NOs concentrations in EBC vary in different airway diseases, and increase with increasing severity of asthma, it is postulated that their measurement may have clinical relevance as a non-invasive biomarker of oxidative-nitrosative stress.

**Nitrites/nitrates**

Nitrite (NO₂⁻) and nitrate (NO₃⁻) are end-products of NO oxidation. Total EBC NO₂⁻/NO₃⁻ concentrations were significantly higher in CF patients and smokers than in healthy controls [36,37]. They were also higher in patients with asthma than in normal subjects [38]. Inhaled steroid
therapy led to decreased levels of NO₂/NO₃ in EBC. A positive correlation between NO₂/NO₃ levels and H₂O₂ concentrations in EBC from asthmatic patients was also observed [38].

Cytokines
Measurement of proteins in exhaled breath condensate is difficult due to very low concentrations of these biomolecules in the condensate. It has been shown by Balint et al. [39] that IL-8 is increased in breath condensate from unstable CF patients. Higher total protein levels were observed in exhaled breath condensate from smokers compared to non-smokers, but tumor necrosis factor-α (TNF-α) did not show any difference [40]. We found undetectable TNF-α in the condensate (A. Antczak, unpublished data). The assessment of proteins in EBC obviously needs more precise and sensitive methods. A proteomics technique is of considerable interest (inverse acute phase proteins observed in EBC - information from dr Ian Adcock, Imperial College, London, UK).

Eicosanoids

Prostaglandins
Prostaglandin E₂ (PGE₂) relaxes airway smooth muscles and exerts potent anti-inflammatory activity [41]. Diminished PGE₂ levels in bronchoalveolar lavage (BAL) obtained from aspirin-sensitive asthmatic patients were observed to further decrease after aspirin administration [42]. Moreover, inhaled PGE₂ can inhibit aspirin-induced bronchoconstriction and urinary leukotriene E₄ (LTE₄) excretion [43]. It is postulated that the failure in PGE₂-breaking mechanism with increased sensitivity to inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) is responsible for overproduction of cys-LTs in aspirin asthma.

PGE₂ levels are neither increased in EBC from asthmatic patients nor decreased in EBC from patients with aspirin-sensitive asthma, thus giving an assumption that PGE₂ deficiency is not responsible for the development of symptoms in aspirin-induced patients [44]. There are no differences in exhaled PGE₂ levels from COPD patients and healthy controls [45].

Leukotrienes
Cysteinyl-leukotrienes (LTE₄, LTC₄, LTD₄) are strong pro-inflammatory products of arachidonic acid metabolism. They are detectable in EBC from healthy subjects and in higher levels in asthmatic patients. Particularly high levels of cys-LTs are observed in EBC from aspirin-induced asthmatic patients. Moreover, they decrease after steroid treatment [44].

As shown by Hanazawa et al. [33] there is further increase in cys-LTs after allergen challenge in mild asthmatic patients. Moreover, withdrawal of steroids in these patients was accompanied not only by clinical deterioration but also by significant increase in cys-LTs levels in EBC.

We have not observed increased LTB₄ levels in asthmatic patients [44]. On the contrary, unstable COPD patients had higher levels of LTB₄ in EBC compared to stable and healthy subjects. They decreased during treatment, however, they were still higher than in healthy subjects [45].

pH
A low pH of exhaled breath condensate has been observed in exacerbation of asthma and COPD [46,47]. This can be associated with increased concentrations of lactic acid as shown in patients with acute bronchitis [48].

CONCLUSIONS
There is accumulating evidence that abnormalities in exhaled breath condensate composition may reflect biochemical changes in airway lining fluid. Detecting and monitoring biomarkers in exhaled breath condensate may be helpful in the diagnosis and follow-up of patients with various pulmonary diseases.

REFERENCES


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