Adenosine in exhaled breath condensate: methodological and clinical aspects

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Introduction

Lower airways can be examined indirectly (for example lung function tests) or directly, but *invasively* (for example bronchoscopy). Exhaled breath condensate (EBC) may be a useful complementary method, since it is *non-invasive* and its components origin from the lower airways. Its principle is that humidity of the exhaled breath will be „condensated“ by leading it through a cooled tube. Level of all kinds of mediators can be measured in the obtained liquid-phase sample. Despite its advantages EBC cannot be used in clinical practice because concentration of molecules in exhaled breath condensate is very low, and therefore their precise measurement is difficult. The question how inhalation during sample collection (which can occur via mouth or nose) influences composition of exhaled air has not answered yet. Wearing nose-clip breathing is allowed only through the mouth, while without nose-clip people can inhale either via nose or through the mouth. At last, it is not clear how much the mouth influences the composition of exhaled breath.

We investigated the level of three mediators in our studies (adenosine, ammonia, thromboxaneB$_2$ – TXB$_2$), but particularly focused on adenosine. Several *in vitro* and murine studies confirmed its role in asthma. It is also known
that its level in bronchoalveolar lavage elevates in asthmatic patients. Therefore – beside methodological studies – we examined its level in EBC of asthmatics. It is well-known that rhinitis develops in about one third of asthmatic people, and that many non-asthmatic rhinitic patients have ongoing inflammation in their lower airways without any clinical symptom. It would be helpful to have a tool which can screen such a subclinical process. EBC seems to be a suitable technique. Therefore we investigated EBC adenosine levels in patients with allergic rhinitis without asthma, and wheather it shows any association with other inflammatory mediator (fractionated exhaled nitric-oxide – FeNO). pH of asthmatic airways becomes acidic. Ammonia may play a role in regulation of airway pH. TXB₂ is a stable metabolite of the pro-inflammatory mediator TXA₂.

Our questions were the following:

Methodological studies
1. Does the mode of inhalation influence the composition and/or volume of exhaled breath condensate?
2. How much does the mouth alter the composition of exhaled breath?
Studies with asthmatic patients and patients with rhinitis

3. Does the EBC adenosine elevate in allergic asthma, and show association with other inflammatory mediator (FeNO)?

4. Does the EBC adenosine increase in non-asthmatic rhinitic patients, and show association with other inflammatory mediator (FeNO)?

5. Does the inhalation via nose have any effect on EBC adenosine in patients with allergic rhinitis?

Methods

Study groups and study design

Methodological studies

To investigate the mode of inhalation we collected two consecutive samples from 25 healthy subjects (24±5 year) (10-10 minutes with a 15 minute break): first by wearing the nose-clip (inhalation via mouth), the second without the nose-clip (inhalation via nose). Volume of the samples, furthermore EBC levels of adenosine, ammonia, TXB2 and salivary amilase (to exclude contamination by the mouth) were measured. We obtained EBC from ten mechanically ventilated patients (4 ARDS, 3 sepsis, 3 postoperatively ventilated, 57±22 year) by attaching the condenser machine
directly to the expiratory limb of the ventilator circuit (upper airways are excluded).

**Studies with asthmatic patients**

<table>
<thead>
<tr>
<th></th>
<th>Healthy patients</th>
<th>Steroid-naive patients</th>
<th>Steroid-treated patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>40</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>age, year</td>
<td>31,8±2,3</td>
<td>32,6±2,5</td>
<td>35,1±3,0</td>
</tr>
<tr>
<td>FEV1 %</td>
<td>91,2±2,1</td>
<td>85,3±1,9</td>
<td>81,6±2,0</td>
</tr>
<tr>
<td>BDP, μg</td>
<td>—</td>
<td>—</td>
<td>1246±152</td>
</tr>
</tbody>
</table>

**Studies with non-asthmatic rhinitic patients**

<table>
<thead>
<tr>
<th></th>
<th>Healthy patients</th>
<th>Patients with allergic rhinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>age, year</td>
<td>25,7±1,2</td>
<td>35,0±2,4</td>
</tr>
<tr>
<td>FEV1 %</td>
<td>96,3±2,4</td>
<td>97,2±2,3</td>
</tr>
</tbody>
</table>

We collected samples from patients with rhinitis twice after each other (with and without nose-clip) according to the desing of the methodological studies.
Collection of EBC

EBC was collected at -20°C by EcoScreen condenser (Jaeger, Germany). Samples were stored at -70°C until the analysis of mediator levels.

Measurement of mediators

FeNO was measured by a chemiluminescence analyzer. EBC adenosine was analysed by HPLC-method, ammonia by spectrophotometry, TXB$_2$ by radioimmunoassay, amylase by ensimatic kinetic technique.

Statistical analysis

We used paired t-test to compare the two modes of sample collection, and one-way ANOVA in all the other cases. Data are given as mean±standard deviation, difference were considered significant at p<0.05.

Results

*Does the mode of inhalation influence the composition and/or volume of exhaled breath condensate?*

We obtained significantly more sample when subjects did not wear the nose-clip (inhaling via nose) as compared to the “traditional” sample collection using the nose-clip (2321±736μl vs. 1746±400μl, p=0.0001, respectively), while mediator levels did not differ in the samples between the two methods (adenosine: 7.9±3.9nM vs. 8.3±4.3nM; ammonia: 74.0±57.0μM vs. 73.9±54.7μM; TXB$_2$: 24.9±18.9pg/ml vs. 24.9±18.9pg/ml).
There was a significant linear correlation in levels of EBC adenosine ($r=0.783$, $p<0.0001$) and ammonia ($r=0.707$, $p<0.0001$), but not in TXB$_2$ ($r=0.364$, $p=0.073$) between the two modes of collection.

**How much does the mouth alter the composition of exhaled breath?**

<table>
<thead>
<tr>
<th>EBC</th>
<th>n</th>
<th>adenosine (nM)</th>
<th>ammonia (μM)</th>
<th>TXB$_2$ (pg/ml)</th>
<th>salivary amilase (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>via mouth</td>
<td>25</td>
<td>8±4</td>
<td>74±55</td>
<td>31±20</td>
<td>8±6</td>
</tr>
<tr>
<td>via intratracheal tube</td>
<td>10</td>
<td>29±23</td>
<td><strong>11±16</strong></td>
<td>44±11</td>
<td>—</td>
</tr>
</tbody>
</table>

Direct contamination by saliva is most likely negligible, because amilase in EBC is approximately 10,000 times less than in saliva ($>10^5$U/l). EBC ammonia was significantly decreased in samples directly obtained from lower airways of mechanically ventilated patients than in EBC of healthy people suggesting that ammonia was added to the exhaled air in the upper airways and/or in the mouth.

**Does the EBC adenosine elevate in allergic asthma?**

EBC adenosine was significantly elevated in steroid-naïve patients with allergic asthma (14.8±7.2nM) as
compared to healthy (9.3±6.4nM, p<0.01) and steroid-treated patients with asthma (10.3±5.6nM, p<0.05). It also showed significant correlation with FeNO (r=0.398, p=0.0081).

Does the EBC adenosine increase in non-asthmatic rhinitic patients?

EBC adenosine was significantly higher in non-asthmatic allergic rhinitic patients than in the healthy control group (12.4±1.3nM vs. 6.5±0.7nM, p=0.0019, respectively) (Figure 1). EBC adenosine showed significant correlation with FeNO (r=0.41, p=0.009, n=42).

Figure 1. Individual EBC adenosine and exhaled NO levels. Both exhaled adenosine and NO were significantly higher in patients with rhinitis than in healthy subjects.
Does the inhalation via nose have any effect on EBC adenosine in allergic rhinitic patients with upper airway inflammation?

Mode of inhalation during sample collection did not effect EBC adenosine in healthy people (6.5±0.7nM inhaling via mouth, 6.8±0.8nM inhaling via nose). However we measured in average of about 50% higher adenosine levels in patients with allergic rhinitis when they inhaled throut the nose than when via mouth (17.7±2.8nM vs. 12.0±1.4nM, p=0.007, respectively) (Figure 2).

**Figure 2.** Adenosine levels in EBC of healthy (A) and rhinitic (B) subjects obtained by inhaling via mouth and nose. Adenosine levels of allergic rhinitis patients was significantly higher in samples obtained by nasal inhalation than by oral inhalation.
Discussion

Methodological studies

More sample can be obtained at the same time if subject inhale through their nose during the sample collection. Although inhalation via nose does not change composition of EBC in healthy people, but it does alter it in patients with upper airway inflammation, therefore it seems more reasonable to condensate everybody in the same way wearing the nose-clip.

Since only negligible amilase activity can be detected in condensate samples, we think that direct contamination with saliva is minimal. This idea is further supported by the adenosine and TXB₂ levels of EBC obtained directly from lower airways of mechanically ventilated patients, because they were in the same range as measured in samples obtained through the mouth in the “traditional” way. Ammonia however was not detectable in EBC collected from the lower airways. This suggests that EBC ammonia was added from the upper airways to the exhaled air – probably by evaporation, since it is a volatile molecule.

Studies with asthmatic patients and patients with rhinitis

Our data show that adenosine in exhaled air is elevated in steroid-naïve asthmatic patients as compared to healthy people. This difference indicates higher number and activity
of inflammatory cells in the lower airways. There was association between concentration of adenosine and FeNO. These observations suggest that exhaled adenosine may be a potential inflammatory marker of the lower airways.

It is known from the literature that co-morbidity of allergic asthma and rhinitis often happens and that many patients with rhinitis have asymptomatic inflammation in their lower airways. According to our findings EBC adenosine is elevated in patients with rhinitis without asthma as compared with healthy subjects suggesting that subclinical inflammation is present in the lower airways of these patients. This hypothesis is supported by the following considerations. First, adenosine concentrations in rhinitis patients were in the same range as found previously in asthmatic patients. Second, adenosine levels correlated significantly with FeNO, a marker of lower airway inflammation.

Interestingly, EBC adenosine in patients with rhinitis is significantly higher when they inhale via nose during the condensation than by oral inhalation. Since this difference was not detected in healthy people, we assume that it was caused by the high adenosine levels of the inflamed nasal cavity. This means that inflammatory mediators from upper airways can enter lower airways by inhalation, and this
“chronic circulation” may contribute to the development of asthma in patients with rhinitis.

**Summary of the new results and conclusions**

*Methodological studies*

Inhalation via nose during condensation significantly increases the volume of the samples, influences the mediators levels in patients with rhinitis, but not in healthy subjects. Therefore nose-clip use is advisable during condensation. Direct contamination by saliva is minimal. Ammonia origins most likely from the upper airways.

*Studies with asthmatic patients and patients with rhinitis*

EBC adenosine levels are significantly increased in steroid-naïve asthmatics, in patients with allergic rhinitis without asthma, show significant correlation with FeNO, suggesting that exhaled adenosine is marker of lower airway inflammation.

Inhalation through the nose significantly increases the concentration of adenosine in exhaled breath in patients with allergic rhinitis. This suggests that inhalation of inflammatory mediators from the nasal cavities into the lower airways can occur.
Acknowledgement

First of all I say thank to my supervisor, Dr. Ildikó Horváth, for her professional help, unselfish approach which allowed me to participate at many international meeting and to make a two-year professional training in America, and especially for her always devoted, always accommodating, joyful personnality. helpful

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Last, but not the least, I am very greatful to Dr. Angela Haczku, who was my supervisor and mentor in Philadelphia during my two-year professional training, and from whom I learned a lot about scientific work.
Articles related to the thesis


Abstracts related to the thesis


Vass G., Huszár É., Barát E., Kiss D., Pénzes I., Kollai M., Horváth I. Comparison of mediator levels in exhaled breath condensate obtained through tracheostomy or orally. Eur Respir J. 2003;22:293s.