

Oxidative stress markers in exhaled breath condensate in inflammatory lung disorders

Doctors thesis

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Introduction

Production of oxidative free radicals and protecting effect of antioxidant mechanisms is continuous in human cells. Balance in oxidant and antioxidant field can easily change in different pathological situation causing oxidative stress and inflammation in different organs.

Inflammation in airways is a well known etiological factor in asthma, COPD or bronchiectasis. Hypoxia- reperfusion mechanism indicate acut lung disorders in ventillated patiens and in patiens undergoing thoracic surgery.

Physical training also cause oxidative stress, consecutive inflammation in the airways in patiens with exercise induced- asthma by leucotriene/ adenosin pathways.

Detection of airway inflammation and sampling of airways can identify the role of different cell types and mediator in lung diseases.

We investigated the role of biomarkers in separate lung disorders as exercise induced asthma and patient after cardiothoracic surgery.

I focused on oxidative stress markers and leucotriene production as potential signal molecules of airway inflammation.

Objectives:

The type of airway inflammation is diverse in the different diseases. The airway inflammation comes along with oxidative stress, a process where mediators are released which certainly contribute to the development of airway diseases in a yet partially understood manner. The collection of exhaled breath condensate is a direct, non-invasive method for sampling airway processes. In my studies different molecules released during airway inflammation (leucotriene, hydrogen-peroxide, 8-isoprostane levels) were determined. In addition to methodological aspects, the pathomechanisms of exercise induced bronchospasm and of responses to mechanical ventilation in thoracic surgery patients were investigated in my studies.

Our goal was to determine the effect of exercise on the concentration of airway cysteinyl leukotriene in asthmatic patients by measuring Cys-LT in exhaled breath condensate (EBC).

Validate an on-line method of H₂O₂ level in EBC and define influencing factors of actual hydrogen-peroxid levels in healthy subjects.

Effect of breathing type on H₂O₂ levels in EBC in healthy subjects.

We compared the analysis of markers of lung injury-hydrogen peroxide and 8-isoprostane- in exhaled breath condensate and bronchoalveolar lavage in endotracheally intubated patients before and after coronary artery bypass graft surgery with cardiopulmonary bypass and lobectomy.

Methods

Subjects in exercise induced asthma study

We studied 17 nonsmoking asthmatic patients (28 ± 10 years, mean \pm SD) with a history of exercise-related dyspnea who were attending the outpatient clinic of National Koranyi Institute for TB and Pulmonology, Budapest, Hungary. All patients met the Global Initiative for Asthma diagnostic criteria for bronchial asthma.. Subjects were atopic, as demonstrated by at least two positive results to skin prick test with the following common aeroallergens: house dust mite, cat dander, dog dander, grass pollen, and *Aspergillus fumigatus*. None of them were receiving inhaled corticosteroids therapy within 2 weeks prior to the study; β 2-agonists were withdrawn ≥ 12 h before the exercise.

We included six healthy, nonsmoking volunteers as a control group (25 ± 6 years).

The research was carried out according to the Declaration of Helsinki. The protocol was approved by the local ethics committee, and written informed consent was obtained from each subject before the study.

Subjects in study with detection hydrogen-peroxide changes in healthy subjects

Sixteen non-smoking, healthy volunteers from the NHS Royal Brompton Hospital Asthma Research Laboratory were recruited (8 males, 8 females, mean age: 32 ± 5.5 years). Subjects had normal lung function values with no history of acute or chronic respiratory diseases or respiratory infection in the previous four weeks. None of them was taking any medication. The protocol was approved by the Ethics Committee of the Royal Brompton and Harefield National Health Service Trust and informed consent was obtained from each subject participating in the study.

Subjects of study with patients after cardiothoracic surgery

Twenty-six patients (21 males; 64 [56–72] years) undergoing CABG surgery and 19 patients (9 males; 62 [57–69] years) undergoing lobectomy for cancer participated in the study. Prior to surgery, all patients gave informed consent for the investigation, the protocol for which was approved by the ethics committee of the Royal Brompton and Harefield Hospitals NHS Trust.

Study design of EIA study

Briefly, first EBC was collected for 5 minutes. This was followed by the measurement of exhaled NO and FEV₁ in each study participant. Participants were then subjected to a standardized, 8-minute treadmill exercise test.

Afterwards, two cycles of EBC collection for 5 minutes immediately followed by FEV₁ measurement was performed. All measurements were performed indoor with fixed temperature and humidity (25 °C, 75%).

Study design of hydrogen-peroxide detection in healthy subjects

In a preliminary study we assessed EBC H₂O₂ concentration at three different time-points of the day (morning: 8:00-9:00 am, noon: 12:00-13:00 and afternoon: 16:00-17:00) to determine optimal sampling time for the main study. All subjects were instructed to refrain from heavy exercise before each collections, but otherwise continue with normal daily schedule. In the morning, EBC condensate was collected after having breakfast. EBC samples were collected during tidal breathing for 10 min. Based on the data obtained afternoon was chosen for sample collection in the main study.

In the main part of the study, subjects were asked to visit the laboratory in the afternoons of three consecutive days. On day one, the intra-subject reproducibility of EBC H₂O₂ measurement was tested. Subjects were asked to perform the same EBC collection procedures twice after each other: they were asked to breathe normally (tidal breathing) into a cooling chamber for 10 min to collect EBC and then to perform a second EBC collection in the same manner. Only approximately one minute long technical break was kept between the two sampling periods (that was required to change the sampling tubes). On day 2 again two sampling periods were performed: first EBC collection with normal tidal breathing was performed and it was followed by the second collection period when subjects were asked to switch to deep and large inhalations and long exhalations with an individually comfortable frequency (breathing with increased tidal volume). On day three the same two EBC collection

periods were repeated in reverse order: first EBC was collected while subjects breathed with increased tidal volume and this was followed by another sampling period with normal tidal breathing. Importantly, breathing with increased tidal volume used in our study is different from maximal voluntary ventilation as it is designed to avoid hypocapnic dizziness or collapse by keeping slow and long expiratory periods. Each EBC collection period lasted for 10 minutes. Between sampling periods only a short technically required break was kept that lasted for approximately one minute. Subjects wore nose-clips during sampling and were advised to swallow their saliva during sampling whenever it was needed.

Study design in patients with cardiothoracic surgery

Hydrogen peroxide, pH, LTB₄, and myeloperoxidase were all measured in EBC, collected from the same patients.⁸ Isoprostane was measured in BAL and EBC samples taken from a different patient group.

CABG Surgery

All operations were performed through a median sternotomy on cardiopulmonary bypass using a membrane oxygenator (D903 Avant; Dideco, Gloucester, UK). Patients were cooled to 32°C. The lungs remained at functional residual capacity during cardiopulmonary bypass.

Lobectomy

After induction of anesthesia, patients underwent rigid bronchoscopy and a double lumen endotracheal tube was sited. To facilitate resection, the lung was deflated and the contralateral lung ventilated. Postoperatively, the endotracheal tube was removed either in the operating room or after transfer to the recovery unit

Collection of EBC with EcoScreen condenser

EBC was collected using the EcoScreen condenser (Jaeger, Hoechberg, Germany).

At rest, subjects breathed at normal frequency and tidal volume. Subjects were instructed not to alter their breathing pattern volume and route of inhalation during EBC collection and to swallow their saliva periodically. There was no significant difference in minute volume between the groups during EBC collection. Collected samples were stored frozen (−70°C) until analysis. Breathing parameters including minute ventilation (V_m), tidal volume (V_t), frequency, expiratory time and flow rate were recorded during each collection period using a tidal breathing analyzer (Masterscreen, Hoechberg, Germany).

Exhaled Breath Condensate Collection with RTube system

EBC was collected from intubated patients before and 30 minutes after surgery. Exhaled breath flowed through the one-way valve into the polypropylene collection tube that was cooled by a surrounding aluminium sleeve at 20°C. (RTube; Respiratory Research Inc., Charlottesville, VA). The fraction of inspired oxygen was 0.4–0.5 and the tidal volume was standardized at 10 ml/kg during EBC collection. Humidification, in the form of a heat and moisture exchanger (Hydro-Therm HME; Intersurgical Ltd., Berkshire, UK) was disconnected from the ventilator tubing a minute before EBC collection was started. Exhaled breath flowed through a one-way valve into the polypropylene collection tube that was cooled by a surrounding aluminium sleeve at -20°C. Preliminary experiments were performed collecting EBC from ventilated patients, the results of which suggested that the yield of EBC (1–2 ml) did not increase substantially and reliably after 15 minutes. The antioxidant, butylated hydroxytoluene was added and the aliquoted samples stored at -70°C.

Lung function test and fractional exhaled nitric oxide (FENO) measurement

FEV₁ was measured by means of an electronic spirometer (PDD-301/s, Piston, Budapest, Hungary) according to the latest guidelines. Three technically acceptable maneuvers were performed and the highest value was used.

FENO levels were recorded with a chemiluminescence analyzer (Model LR2000, Logan Research, Rochester, UK) without wearing a nose-clip by slow exhalation (250 mL/s) from total lung capacity for 20–30 seconds against a resistance (5cm H₂O) to prevent nasal contamination. Three successive recordings were made and the mean of these values was used in all calculations.

Exercise challenge

Subjects exercised on an electrically driven treadmill at room temperature wearing a nose-clip. The speed, gradient, time of exercise and heart rate were displayed continuously during running. Exercise intensity was ramped over 2 minutes to 80–90% of predicted heart rate (220-age) and sustained for 6 minutes. A greater than 15% drop in FEV₁ because of exercise was used as a criterion for a positive BHR test.

BAL Fluid Collection

BAL was performed, after EBC collection, using buffered normal saline (3 aliquots of 60 ml), in different segments of the right middle lobe before and after cardiac surgery. The fluid was centrifuged, cytocentrifuge slide preparations were made, stained with May-Grunwald-Giemsa and differential cell counts performed on at least 300 cells in random fields.

Determination of Cys-LT

Cys-LT concentrations were measured by using a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The detection limit was 13 pg/mL. The assay was validated for measurements in EBC and calibration curves were created in the buffer solution provided by the manufacturer.

H₂O₂ measurement by on-line detection method

On-line H₂O₂ measurement was performed by the EcoCheck™ biosensor system (FILT GmbH, Berlin, Germany) by a validated method as previously described (15). Briefly, the measurement is based on an electron transfer process catalysed by peroxidase that is fixed on the surface of the biosensor. Peroxidase-induced H₂O₂ reduction results in electron transfer on the biosensor that is measurable as a potential difference. The degree of electron transfer is dependent on the H₂O₂ concentration in the sample. H₂O₂ concentration was measured from fresh samples immediately after collection. Each detection procedure lasted 8 minutes.

Hydrogen Peroxide Analysis by colorimetric method

Hydrogen peroxide was measured in EBC (0.5 ml) using a colorimetric method. Values in EBC were calculated from a standard curve prepared from stock standardized hydrogen peroxide solution (Sigma, Poole, UK).

pH Measurement

pH measurements (Jenway 350 pH meter; Spectronic Instruments, Leeds, UK) were performed immediately after collection of EBC (0.5 ml), after deaeration with argon (350 ml/minute for 10 minutes).

Leukotriene B₄ and Myeloperoxidase Assays

Specific enzyme immunoassays for LTB₄ (Cayman Chemical, Ann Arbor, MI) and myeloperoxidase (Calbiochem-Novabiochem Co., San Diego, CA) were performed on EBC (0.1 ml). The intraassay and interassay variabilities were less than 10%. The detection limit of the assays was 7.8 pg/ml for LTB₄ and 1.5 ng/ml for myeloperoxidase.

Statistical analysis for EIA

Results are expressed as a mean ± SD and for FE_{NO}, FE_{NO50}, and Cys-LT concentration median/range/. Normality was tested with Shapiro–Wilk normality test. Because Cys-LT concentrations and FE_{NO} levels were not normally distributed, these values were logarithmically transformed and log transformed data were used for further analysis. Repeated measures ANOVA and two-way ANOVA together with Bonferroni post hoc test were used to compare baseline versus post-exercise and asthmatic versus healthy and EIB versus non-EIB subgroup data, respectively. Pearson test was performed to analyze the correlation between the different

variables t-Tests were used to compare asthmatic versus control groups and EIB versus non-EIB subgroups. The maximal increase/decrease in Cys-LT concentration and FEV₁ was calculated by using the highest/lowest post-exercise value and plotted as percentage of the baseline value. P-values < 0.05 were considered significant.

Statistical analysis in hydrogen-peroxide detection

Data are expressed as mean ± SEM. As data showed normal distribution, to assess daytime changes in H₂O₂ concentration repeated measures of ANOVA and to compare different breathing patterns paired Student's t-test was used. Correlation coefficients were calculated by the Pearson's method. Repeated measurements were analyzed by the Bland-Altman test and the calculation of the coefficient of variation. All calculations were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). A p value < 0.05 was considered statistically significant.

Statistical Analysis of parameters in cardiathoracic surgery

Data were analyzed using GraphPad Prism version 3.00 (GraphPad Software, San Diego, CA). Data were not normally distributed (as assessed by the Kolmogorov-Smirnov test) and groups were compared using the Mann-Whitney test. Correlation coefficients were determined using Spearman's rank correlation test. All data are expressed as median and interquartile range. Significance was defined as a p value less than 0.05.

Results

Cys-Leukotriene levels in exercise induced asthma

Baseline comparisons between asthmatic and healthy groups

There was no difference in the baseline FEV₁ percentage predicted between the asthmatic and healthy groups (99 ± 11% pred. vs. 95 ± 3% pred., p = 0.33). However, baseline Cys-LT concentration (157 pg/mL /43–400/ vs. 69 pg/mL /28–141/, p = 0.03) was significantly higher in asthmatic subjects compared with healthy individuals but no significant difference was seen in FENO between the two groups (8.8ppb vs. 5.8ppb, p = 0.10).

Effect of exercise on FEV₁ and Cys-LT concentration

As a result of the exercise challenge, FEV₁ dropped from that of the baseline value (−9% ± 13%), yet there was no significant change in the time course of FEV₁ in the asthmatic group

(n = 17, p = 0.20). In the healthy group not significant, increase in FEV₁ was observed (n = 6, p = 0.60, 4 ± 5%). However, in asthmatic patients exercise caused an increase in EBC Cys-LT levels (n = 17, p = 0.03, repeated measures ANOVA) with no difference between timepoints evaluated by a post hoc test (130pg/mL /42–410/ and 180pg/mL /58–1000/, 0 and 10 minutes after exercise, respectively). On the contrary, no change was found in healthy subjects (n = 6, p = 0.34; 68pg/mL /52–147/ and 93pg/mL /25–140/, 0 and 10 minutes after exercise, respectively). There was a significant difference in the time course of changes in Cys-LT concentrations between asthmatic and healthy control groups (p = 0.02, two-way ANOVA).

Relation between FE_{NO} levels and exercise-induced changes in FEV₁ and Cys-LTs

Although there was a strong negative correlation between baseline FE_{NO} values and the maximal decrease in FEV₁ in asthmatic group (n = 17, r = -0.77, p < 0.001), no correlation was found in healthy volunteers (n = 6, r = -0.76, p = 0.07). Furthermore, a positive correlation was found between the baseline FE_{NO} levels and the maximal increase in Cys-LT concentration in the asthmatic group (n = 17, r = 0.57, p = 0.01) with no relation observed in the healthy subjects (n = 6, r = 0.27, p = 0.60).

Comparisons between the EIB and the non-EIB subgroups

EIB developed in 41% of patients (n = 7, EIB group), with no significant bronchoconstriction occurring in the rest of the patients (n = 10, non-EIB group). There was no significant difference between the EIB and the non-EIB groups regarding baseline FEV₁% predicted (98 ± 14% pred. and 99 ± 10% pred., p = 0.47 and p = 0.87) and baseline EBC Cys-LT concentrations (90pg/mL vs. 158pg/mL, p = 0.46). However, FE_{NO} was significantly higher in the EIB group compared with the non-EIB group (15.9ppb vs. 6.1ppb, p < 0.001). FEV₁ significantly decreased in the EIB group compared with baseline value (-23% ± 6%, p = 0.004). In the non-EIB group FEV₁ showed a slight, but nonsignificant, increase (7 ± 7%, p = 0.16). There was no difference in the time course of changes in Cys-LT concentrations between the two subgroups (p = 0.52, two-way ANOVA); however, there was a trend toward an increase in the EIB group (EIB group: 130pg/mL /42–180/ and 150 pg/mL /58–1000/, 0 and 10 minutes after exercise, respectively, p = 0.06; non-EIB group: 141 pg/mL /74–410/ and 190 pg/mL /95–320/, 0 and 10 minutes after exercise, respectively, p = 0.47, repeated measures ANOVA). The highest post-exercise Cys-LT concentrations were 1000 pg/mL and 410 pg/mL in the EIB and non-EIB groups, respectively. The maximal increase in mediator concentration

was significantly higher in the EIB group than in the non-EIB group ($111 \pm 108\%$ vs. $21 \pm 46\%$, $p = 0.03$).

Changes of hydrogen peroxide concentration in exhaled breath condensate in healthy subjects with different type of breathing

Circadian change in EBC H₂O₂

In the preliminary study we observed a significant increase in hydrogen peroxide level during the day. The difference between morning – lunchtime, morning - afternoon and lunchtime-afternoon levels were all significant (554 ± 51 nmol/l; 738 ± 61 nmol/l, $p < 0.01$; 1124 ± 136 nmol/l, $p < 0.001$ vs. morning value and $p = 0.008$ vs. noon value, respectively).

Sampling in the afternoon period was chosen for the main part of the study.

Reproducibility of EBC H₂O₂ measurement

The peroxide concentration of EBC collected during tidal breathing on the two consecutive sampling was not significantly different (888 ± 176 nmol/l vs. 874 ± 156 nmol/l). There was a strong correlation between readings ($r^2 = 0.98$; $p < 0.0001$;).

Effect of breathing type on breathing parameters

The type of breathing had an obvious effect on various breathing parameters . Accordingly, V_m, V_t, expiratory time and flow rate were significantly increased and breathing frequency decreased when subjects were asked to perform breathing with increased tidal volume.

Effect of breathing type on EBC volume and H₂O₂ concentration

EBC volume was significantly increased during breathing with increased tidal volume. In parallel, H₂O₂ concentration in samples obtained during this type of breathing was significantly decreased. H₂O₂ level was decreased in all subjects. Assessment of the inter-subject variability of H₂O₂ concentration in samples obtained during normal breathing and breathing with increased tidal volume revealed a coefficient of variation of 49 and 54%, respectively.

When sampling was repeated in reverse order similar results were obtained. EBC H₂O₂ concentration was lower in samples obtained during breathing with increased tidal volume compared to that of EBCs collected with normal tidal breathing (778 ± 145 nmol/l vs. 1298 ± 151 nmol/l ; $p < 0.005$).

Correlations between breathing parameters, EBC volume and H₂O₂ concentration

There was a significant positive correlation between V_m and EBC volume both during normal tidal breathing ($r=0.693$, $p<0.005$); breathing with increased tidal volume ($r=0.841$, $p<0.001$). Similarly, expiratory flow rate showed a significant correlation with EBC volume in both types of EBC sampling (normal breathing: $r=0.632$, $p<0.01$; breathing with increased tidal volume: $r=0.873$, $p<0.001$).

In contrast, there were no correlations between H₂O₂ concentration and V_m or expiratory flow rate either during normal tidal breathing or breathing with increased tidal volume. Similarly, no associations were found between V_t or frequency and EBC volume or H₂O₂ concentration. EBC volume did not correlate with H₂O₂ concentration either, irrespectively of the type of breathing .

Oxidativ stress markers in exhaled breath condensate after cardiathoracic surgery

None of the patients in this study satisfied the criteria for ALI/ARDS or required mechanical ventilation for more than 24 hours after surgery. Neither BAL nor EBC collection was associated with adverse effects.

Comparing hydrogen-peroxide levels in exhaled breath condensate

Greater postoperative EBC hydrogen-peroxide concentrations were measured in patients undergoing lobectomy than in those undergoing cardiac surgery (**0,2** (0,03-0,5) μ M-**0,8** (0,15-1,9) μ M; $n=19$; $p=0,03$).

There was no difference in preoperative EBC hydrogen peroxide concentration in patients undergoing CABG surgery comparing patients with lobectomy. Although levels of hydrogen-peroxide in EBC did not even change in postoperative cases in patients after CABG surgery.

Comparing 8- isoprostane levels

However, there was no significant difference in EBC 8-isoprostane measured as **11** (7–25) pg/ml pre- and **10** (7–13) pg/ml postoperatively.

8-Isoprostane levels were similar in both groups preoperatively.

pH levels

pH level significantly decreased (6 (5,7–6,4) preoperatively, 5,5 (5,3–6) postoperatively(n=19; p=0,02)) in patients with lobectomy.

Levels in pH even decreased but not significantly in EBC after CABG surgery. (n=26; p=0,1).

Markers of lung inflammation after CABG surgery

After lobectomy Hydrogen peroxide concentration in EBC rose from 0.2 (0.03–0.5) uM pre- to 0.8 (0.15–1.9) uM postoperatively (n = 19; p =0.03)

In cardiac surgery groups, there was no difference in preoperative EBC hydrogen peroxide concentration (p = 0.32) and pH (p= 0.92), but lower preoperative EBC LTB4 concentrations were measured in patients undergoing lobectomy (p = 0.04). There was no difference between the groups in postoperative EBC LTB4 concentration (p= 0.14) or pH (p= 0.19). However, greater postoperative EBC hydrogen peroxide concentrations were measured in patients undergoing lobectomy than in those undergoing cardiac surgery (p =0.008). 8-Isoprostane levels were similar in both groups pre- and postoperatively.

Conclusions

I. We demonstrated that EBC Cys-LT concentration was elevated minutes after the exercise and there was a positive correlation between changes in Cys-LT levels and the baseline exhaled NO concentration in asthmatics whereas no similar findings were observed in healthy volunteers. We found a significant correlation between baseline FENO and the maximal change in FEV1 after exercise. FENO in asthma is regarded as a marker of eosinophilic airway inflammation. This inflammation might also explain the relationship between baseline FENO and the magnitude of post-exercise change in Cys-LT concentration. However, only 41 percent of asthmatics developed EIB on exercise testing. Comparing the EIB and non-EIB subgroups of asthmatics, we found higher FENO level in the EIB-positive subgroup.

II. We demonstrated a reliable method for detection of H₂O₂ in exhaled breath condensate. This on-line/ biosensor analytical method has a good accuracy and reproducibility for condensate samples. We have shown a circadian variation on level of

hydrogen peroxide in EBC. H_2O_2 was detectable in each condensate samples the lowest level was measured in morning hours.

III. Breathing pattern does influence hydrogen peroxide levels in EBC samples collected over different breathing type. We found lower H_2O_2 levels during collection with deep inhalations compared with those levels we found with normal tidal breathing collection. Our results contribute to standardisation of circumstances in EBC collection.

IV. We measured increased hydrogen peroxide and LTB_4 and significantly decreased pH levels after lobectomy but not in CABG patients. These oxidative stress markers are detectable in EBC samples even in ventilated patients and breath condensate collection can give good informations over inflammatory process before clinical symptoms appear. EBC collection is easy to use and non-invasive method for sampling and monitoring inflamed airways in intensive care units.

Publications

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