ERJ Express. Published on September 13, 2006 as doi: 10.1183/09031936.00151905

Saliva is one likely source of leukotriene B_4 in exhaled breath condensate

Flora Gaber, Fernando Acevedo,

Ingrid Delin, Britt-Marie Sundblad, Lena Palmberg, Kjell Larsson, Maria Kumlin

and Sven-Erik Dahlén

Division of Physiology, the National Institute of Environmental Medicine,

Karolinska Institutet, SE-17177 Stockholm, Sweden

Short title:
LTB_4 in EBC is likely a saliva contamination

Corresponding author:

Flora Gaber

Unit for Experimental Asthma and Allergy Research

Division of Physiology, P.O. Box 287

The National Institute of Environmental Medicine

Karolinska Institutet

SE-171 77 Stockholm, Sweden

Phone: +468 5248 7571

Fax: +46 8300 619

E-mail: Flora.Gaber@ki.se

Supported by: The Swedish Heart and Lung Foundation, the Swedish Medical Research Council, the Swedish Farmer Foundation for Agricultural Research, the Swedish Asthma and Allergy Association, and Karolinska Institutet

Copyright 2006 by the European Respiratory Society.

Abstract

Leukotriene B_4 (LTB_4) in exhaled breath condensate (EBC) has been reported to be elevated in airway inflammation. The origin of leukotrienes in EBC is however not established.

AIMS

To measure LTB_4 levels in EBC collected in two challenges characterized by a strong neutrophilic airway inflammation. To compare LTB_4 levels in EBC with levels in sputum and saliva.

METHODS

Leukotriene B_4 and α-amylase were measured in EBC from 34 healthy subjects, exposed in a swine confinement building or to a lipopolysaccharide provocation. These markers were also measured in induced sputum in 11 of the subjects. For comparison, LTB_4 and α-amylase were measured in saliva from healthy subjects.

RESULTS

Only four out of 102 EBC samples had detectable LTB_4 (28-100 pg/mL). Alpha-amylase activity was detected in the LTB_4 positive samples. In contrast, LTB_4 was detected in all examined sputum supernatants in the same study (median 1190 pg/mL). The median LTB_4 level in saliva was 469 pg/mL.

CONCLUSION

High levels of LTB_4 in saliva, and presence of LTB_4 in EBC only when α-amylase was detected, indicates that LTB_4 found in EBC is due to saliva contamination. As LTB_4 consistently was present in sputum supernatants, EBC may be inappropriate for monitoring airway LTB_4.

Key Words

α-amylase, Exhaled Breath Condensate, Induced Sputum, Leukotriene B_4, Neutrophilic inflammation, Pig house

Introduction

Exhaled Breath Condensate (EBC) has gained considerable attraction as a new non-invasive technique for measuring markers of airway inflammation (for review see (1)). The procedure normally involves tidal breathing for 5 to 15 minutes and passage of the expired air through a cold trap where the fluid phase is condensed. A great number of mediators, markers and other molecules have been measured in EBC (1). Following the original description of the method by Russian investigators in 1980 (2), there are as of date more than 300 papers published on the topic, and more than 200 of those are from the last five years. The method is thus very attractive but as pointed out in the recent report from an ERS Task Force (1), there are methodological issues that remain unresolved. In fact, there are few experimental studies that satisfactory explain the processes that determine the transfer of different molecules from the lungs and airways into condensates of exhaled air.

Leukotriene B_4 (LTB_4) is a potent chemotactic mediator implicated to be involved in several inflammatory reactions in the lungs and airways (3, 4). Leukotriene B_4 is predominantly produced by activated neutrophils, but also by alveolar macrophages and in transcellular reactions involving inflammatory cells and surrounding structural elements (4). In addition to recruiting neutrophils and causing increased microvascular permeability (5), LTB_4 may stimulate lymphocyte migration in the lung (6) and constriction of pulmonary blood vessels via indirect mechanisms (7). In comparison with healthy subjects, the levels of LTB_4 have been reported to be elevated in EBC collected from subjects with asthma (8), cystic fibrosis (9) and chronic obstructive pulmonary disease (COPD) (10).

The primary aim of the study was to measure the levels of immunoreactive LTB_4 in EBC collected in a human model where a pronounced neutrophilic inflammation is induced (11). The model involves a standardised exposure of healthy subjects in a pig house for three hours. This challenge results in a 2-3 fold increase in bronchial responsiveness to methacholine, and an associated intense airway neutrophilic inflammation. Previous studies have shown a 75-fold increase in the number of neutrophils in bronchoalveolar lavage fluid (11) and a 2 to 5.5-fold increase in the levels of LTB_4 in the nasal lavage fluid following exposure in a pig house (12, 13). It was therefore considered suitable to use the non-invasive EBC method to measure LTB_4 levels in a study where responses to pig house dust and lipopolysaccharide (LPS) were to be characterised. For comparison, levels of immunoreactive LTB_4 in induced sputum were measured in a subgroup of the subjects. In addition, saliva was collected at baseline from another group of healthy subjects and immunoreactive levels of LTB_4 as well as α-amylase activity were measured.

Material and Methods

Subjects

Thirty-four healthy subjects, mean age 38 (range 22 - 61) years, participated in the study. None of the subjects had a history of asthma, allergy or other lung diseases and none had a respiratory tract infection during the last week prior to the study.

Saliva was collected from another group of 17 healthy non-smoking, non-atopic, non-asthmatic volunteers, mean age 31 (range 22 – 55) years.

All subjects gave informed consent to participate in the study approved by the Ethical Review Board at Karolinska Institutet.

Study design

All subjects (n=34) were exposed to swine house dust in a pig confinement building while weighing pigs for 3 hours, and on a separate day provoked with a LPS solution (53 μg) using a dosimeter controlled jet nebuliser (Spira Elektro 2, Intramedic, Bålsta, Sweden). Pig house exposure and LPS-challenge were performed in random order separated by 2-5 weeks. Collection of EBC was performed 2-14 days before the first exposure and 6 hours after start of swine house dust exposure and LPS provocation. Induced sputum was collected at baseline, and within 9 hours after start of pig house exposure.

Five ml of saliva at baseline was obtained from another group of healthy volunteers (n=17) by chewing on a plastic tube stopper.

Collection of exhaled breath condensate in a siliconised glass condenser

In a preliminary set of experiments, we tried the ECoScreen (Jaeger, Wurzburg, Germany) for collection of EBC, but found LTB_4 in the EBC only occasionally. It has been reported that the coating of the condenser might be important for the recovery of non-volatile compounds such as eicosanoids (14). Therefore, in our study we used a silicon coated glass condenser to minimize possible binding of the leukotrienes to the coating (Figure 1). The subjects breathed through a mouthpiece (standard cardboard mouthpiece, Vitalograph®, Vitalograph GmbH, Hamburg, Germany) connected to a 32 mm pvc tube with a 90° bend coupled to the inlet of a vertically placed condenser tube. The diameter of the inlet (approx. 20 mm) was smaller than the pvc, allowing a gap which functioned as a saliva trap. The junction was tightened with laboratory film (Parafilm "M"®, Pechiney plastic packaging, Chicago, IL, USA). The condenser, 20 cm long, was of glass type Allihn, pre-treated with silicon, and refrigerated at 0.5°C by a thermostatic circulator (LKB, Bromma, Sweden). EBC was collected in an ice chilled 50 mL polypropylene tube. Approximately 5 mL of EBC were collected during 20 min of tidal breathing. Nose clips were not used. The condensate was immediately aliquoted (0.5 mL/aliquot) into 0.75 mL cryotubes (BioStor™ with separate "O"-ring screw caps, National Scientific Supply Co, Inc., Claremont, CA, USA) and stored in -20°C until analysis. The condenser was thoroughly rinsed with distilled water for 3 minutes after each collection.

Induced sputum and sputum processing

In a subgroup of eleven healthy non-smoking subjects, sputum induction and processing were performed as described (15), with minor modifications. The subjects were pre-treated with 400µg inhaled salbutamol (Ventoline®, GlaxoSmithKline AB, Mölndal, Sweden) to inhibit excessive airway constriction. A few subjects could expectorate

sputum spontaneously and the rest (vast majority of the subjects) were induced with an aerosol of isotonic and hypertonic saline, inhaled from an ultrasonic nebuliser (Ultra Neb 2000, DeVilbiss Health Care, Somerset, PA, USA). The nebuliser output was 3 ml/min. The concentrations of saline were 0.9, 3, 4 and 5%; the duration of each inhalation concentration was 7 minutes followed by FEV_1 measurement. After each period of inhalation the subject was instructed to blow his nose and to rinse his mouth with water to minimize contamination of the sputum sample. The subject was asked to cough deeply and to do an attempt to expectorate sputum. The sample was considered adequate when macroscopically appeared to be free from saliva and weighing at least 1000 mg. There was thus no difference in the mean volume of collected sputum before or after swine house dust exposure.

The colour and the weight of the entire sputum were determined. An equal volume of dithiothreitol (DTT, Sputolysin® Reagent, EMD Biosciences, Inc., San Diego, CA, USA) 0.1% was added to the sample for a final concentration of 0.05% DTT and rocked for 15-25 minutes in 37°C water bath for homogenization and to dissociate disulfide bonds. The sample was centrifuged (10 minutes at 280 x g) and the supernatant dispensed into several aliquots, which were kept in -70°C until analysis.

Saliva sampling and processing

Subjects were asked to wash their mouth with water before saliva collection. The sample was centrifuged at 1500 x g for 15 min and the supernatant was stored in -70°C until analyzed. Leukotriene B_4 levels and α-amylase activity was measured as described below.

Measurements of immunoreactive LTB_4

Leukotriene B_4 measurements were performed with an LTB_4 specific enzyme immunoassay as described by the manufacturer (Cayman Chemical Company, Ann Arbor, MI, USA), in duplicates of serially diluted samples. The assay detection limit was 3.9 pg/mL. In all samples, the intra-assay variation was <10.0% and inter-assay variation <12.0%. Analysis was performed within 3 and 5 months of EBC collection, and within 4 months of saliva collection.

Immunoreactive LTB_4 levels were measured in the sputum supernatant as described by the manufacturer, with the modification that DTT of the same concentration as in the sputum supernatant (0.05% DTT) was added to the standard curve and EIA buffer. In the modified assay, the intra-assay variation was 1.9 % and inter-assay variation 4.6 %.

Measurements of amylase activity

Analysis of amylase activity in undiluted EBC samples was performed within 3 and 5 months after EBC collection. The analysis was performed after modifications of the method found at the Calzyme Laboratories, Inc. website (16). Thus, 50 µL of undiluted EBC samples in a double set, or 50 µL saliva samples in serial dilution from 1:100 to 1:12800 in a double set, or 50 µL sputum supernatant samples in serial dilution from 1 to 1:400, or 50 µL α-Amylase standard (Sigma-Aldrich Inc., St. Louis, MO, USA) in serial dilution from 10 to 0.078 U/mL (the limit of detection was thus 0.078 U/mL), or 50 µL 20 mM glucose solution in serial dilution from 20 to 0.15 mM, respectively, were added on a 96-well plate (Nunc A/S, Roskilde, Denmark). Starch solution (1%) in a volume of 50 µL, was added to all wells except for those containing glucose and one set of samples (the difference between samples with and without added starch accounted for the endogenous blank). The plate was left at room temperature (RT) for 10 minutes,

followed by addition of 100 µL of colour reagent, (a mixture of dinitrosalicylic acid and sodium-potassium-tartrate tetra-hydrate, both from Sigma-Aldrich Inc., St. Louis, MO, USA). The plate was sealed (Plate sealer, In Vitro AB, Stockholm, Sweden) and placed in a boiling water bath for 12 minutes. Finally, the plate was read at RT in a spectrophotometer at 540 nm and the amount of α-Amylase was calculated. The intra-assay variability was 2.8% and inter-assay variability was 14.8% (n=5).

Stability test

The stability of LTB_4 in exhaled breath condensate and in saliva was studied. Aliquots of EBC were supplied with 50 pg/mL of synthetic LTB_4 (Cayman Chemical Company, Ann Arbor, MI, USA) and with saliva (1%). The samples were stored in RT. Aliquots were placed in -70°C immediately after LTB_4 addition, as well as after 1h, 2h, 7h and 24h. Aliquots were also stored in -20°C for a long term stability study, and LTB_4 immunoreactivity was measured at regular intervals for 100 days.

Spiking of EBC with increasing amounts of saliva

Exhaled breath condensate (5mL) and saliva (5mL) was collected from one healthy subject. Increasing amounts of saliva was added to EBC aliquots and immunoreactive LTB_4 as well as α-amylase activity was measured as described above.

Statistical analysis

Data are expressed as medians (range) and statistical calculations were performed using Spearman rank test.

Results

Immunoreactive LTB_4 in EBC of healthy subjects exposed in a pig house and to LPS

Exhaled breath condensates (n=102) from altogether 34 subjects examined at three occasions, *i.e.* before and after exposure in a swine house and after LPS provocation (in a cross-over study), were examined for LTB_4 immunoreactivity. Irrespective of when collected, only four out of the 102 EBC samples tested showed immunoreactive LTB_4 levels above the detection limit (3.9 pg/mL), with levels ranging from 28 to 100 pg/mL (median 46.5 pg/mL). Alpha-amylase activity was detected in only 5 of 102 samples: the four samples with detectable LTB_4 and in an additional sample (low α-amylase activity) with LTB_4 below the detection limit (Fig 2). In these five samples, the α-amylase activity ranged from 1.2 to 28.0 U/mL (median: 3.2 U/mL). There was a strong correlation between immunoreactive LTB_4 levels and α-amylase activity in the EBC samples (r^s=1.00, p=0.017, n=5) (Fig 2).

Immunoreactive LTB_4 and α-amylase activity in sputum of healthy subjects exposed in a pig house

In the 11 healthy non-smokers who were exposed in a pig house for three hours, the median level of immunoreactive LTB_4 in the sputum supernatant (pre-and post exposure) was 1190 pg/mL (range 193-5290 pg/mL, n=22) (Fig 3). The median α-amylase activity was 22.3 U/mL (pre-and post exposure, range 0.9-257.7 U/mL, n=22). Only one EBC sample from these subjects contained detectable LTB_4 levels (Fig 3).

Stability of LTB_4 in EBC and saliva

As measurements of immunoreactive LTB_4 were done after storage in the freezer (-20°C), the stability of immunoreactive LTB_4 under conditions relevant to the study was investigated. Immunoreactive LTB_4 levels were stable for 24h in room temperature in EBC and saliva (Fig 4A), and more than 3 months when kept at -20°C (Fig 4B).

Immunoreactive LTB_4 levels and α-amylase activity in saliva

In order to confirm that saliva contamination may add LTB_4 to EBC samples, saliva was collected in 17 healthy volunteers. Immunoreactive LTB_4 levels in saliva were 469 (140-1358) pg/mL. Alpha-amylase activity in the same samples was 426 (147-807) U/mL.

Finally, spiking EBC with increasing amounts of saliva produced consistent parallel increments in LTB_4 levels and α-amylase activity, respectively (Fig 5).

Discussion

We were unable to consistently detect leukotriene B_4 (LTB_4) in 102 samples of exhaled breath condensate (EBC) collected at three occasions from thirty-four volunteers. In contrast, we measured high levels of LTB_4 in sputum supernatants collected from eleven subjects before and after swine house dust exposure in the same study. In a parallel experiment, LTB_4 levels in saliva were found to be between 140 and 1358 pg/mL in healthy volunteers. By the use of a sensitive method for measurement of α-amylase in EBC, we found that all samples that were positive for LTB_4 also contained α-amylase. Therefore, we conclude that saliva is the possible source of LTB_4 in EBC.

The levels of LTB_4 in EBC have been reported to be increased in several respiratory diseases such as asthma, COPD (17) and cystic fibrosis (9). The levels reported are generally in the 10-130 pg/mL concentration range, although in more recent publications the data are expressed as amounts of obtained LTB_4 during a defined time period of tidal breathing (18). The vast majority of published papers report on LTB_4-like immunoreactivity (LTB_4-IR) by the use of enzyme immunoassay (EIA) measurements, but recent papers from one group have validated the measurements of LTB_4 with mass spectrometry (18). There is thus no doubt that LTB_4 may be present in EBC.

The question is, however, what the source of LTB_4 in EBC is. It is conceivable that material from pharynx, the nasal cavity and the mouth may contaminate air from the lower airways before exhalation. Many investigators that measure leukotrienes in EBC have considered this and therefore tested EBC for presence of α-amylase, an enzyme produced by salivary glands (19). An α-amylase activity below the detection limit of the particular assay used, around 20 U of α-amylase activity per mL of EBC

(20), has been taken as evidence that there is no significant salivary contamination of the EBC, and that the EBC sample exclusively contains material from the lower airways. Our results however question whether measurement of α-amylase in EBC with standard assays is sufficient to exclude saliva contamination of the EBC sample. We measured levels of LTB_4 (range 140-1358 pg/mL) and α-amylase activity (range 147-807 U/mL) in saliva in a group of healthy volunteers (n=17). Also with another method, α-amylase activity in saliva was found to be highly variable (range 3-423 U/mL, n=75) (21). Thus, the amylase method originally developed for measurements in saliva is not adequate to detect saliva contamination in EBC. We therefore developed a more sensitive assay with a detection limit of 0.078 U/mL. As shown by our spiking experiments (Fig. 5), already 0.5-1% saliva addition gives rise to increased α-amylase and LTB_4 in EBC samples. This effect is strikingly increased with increased saliva.

In the present study, we collected EBC from a group of healthy volunteers exposed in a pig house, which induces an intense neutrophilic inflammation with 70- and 20-fold increment of neutrophils in BAL and NAL fluid, respectively (22). The levels of LTB_4 in NAL has been found to increase between 2.5 to over 5 times after exposure (12, 13) and IL-8 about 7 to 8 times following exposure in a pig barn (12, 22). The eleven healthy non-smoking volunteers in our study had high levels of LTB_4 in sputum samples, approximately 1000 pg/mL. Nevertheless, we were unable to detect LTB_4-IR in all but one of the EBC samples collected from these subjects.

A stability test revealed that the levels of immunoreactive LTB_4 in EBC are stable for 24 hours in room temperature and at least 3 months in -20°C. We also investigated if saliva in EBC caused a difference in LTB_4 stability. We found no difference between samples supplied with only LTB_4 and samples supplied with LTB_4 and saliva (1%). Thus, the storage of EBC samples for some months until analysis does not appear to

alter the outcome of the LTB_4 assay. Others have claimed that the immunoreactivity of leukotrienes in EBC decreases after 3 weeks of storage (17). However, in that particular study, the LTB_4 levels were dropped to 92% of the original immunoreactivity after the third week, which was within the range of the reported inter-assay variability of 15%.

There is data supporting the hypothesis that turbulent flow enhances recovery of some compounds in EBC (1). The differences in LTB_4 levels in EBC between different patient groups might also reflect events in the upper airways and oral cavity. LTB_4 is the major leukotriene produced in the nasal mucosa (23), and it has been proposed that there is a significant co-morbidity between asthma and rhinitis. One study reported higher leukotriene levels in EBC in patients with seasonal allergic rhinitis as compared with controls. (24). Another factor could be that most patients with COPD are smokers and smokers have increased prevalence of periodontitis. Interestingly, in a recent study, the levels of LTB_4 in EBC of smokers without COPD was found to be high (17). There are also indications from measurements of other substances that the oropharyngeal tract contributes to the contents of EBC. Thus, it was recently shown that nitrite from saliva is an important contaminant of EBC collections (25).

Taken together, our data support the hypothesis that LTB_4 in EBC from healthy volunteers predominantly derives from saliva. Low or undetectable levels of LTB_4 in EBC from healthy controls have been reported, but elevated levels in subjects with different lung diseases. We did not detect LTB_4 in approximately 100 EBC samples, whereas we measured high levels of LTB_4 in induced sputum samples collected before and after the exposure. As we in preliminary studies (see Methods) also tried the ECoScreen condenser with similar negative results, our data are unlikely related to the collection system. Our findings therefore question the appropriateness of EBC as a relevant sample to assess mediators in the lower airways, at least in the case of LTB_4.

We do not however question the theory that LTB_4 could be formed in the lower part of the airways since sputum samples showed a high neutrophil content (26) and LTB_4 concentration after exposure in a pig house, but as others have concluded (25) the aerosol particles that are proposed to transport LTB_4 from the airways may to a much greater extent remain in the exhaled air when formed in the very proximal (including the oropharyngeal tract) rather than the peripheral airways.

In conclusion, our findings support that the presence of LTB_4 in EBC most likely is due to a salivary contamination. The differences in levels of LTB_4 in EBC that have been reported between different groups of patients or between patients and healthy controls, are generally relatively modest and may as discussed be explained by other mechanisms than true differences in recovery of LTB_4 from the lower airways. It remains to be seen if our conclusion only applies to measurement of LTB_4 in EBC, but we hypothesize that salivary origin of material in EBC is likely to be a major confounding factor for all compounds that are secreted in relatively high quantities in the saliva. In order to test this possibility, it is felt that future measurements in EBC need to include a sensitive α-amylase assay and measurements of the particular compound studied also in saliva. It is apparent that many publications reporting levels of leukotrienes in EBC do not present values on α-amylase activity.

Acknowledgements

The authors wish to thank Dr. Alexandra Ek, Marianne Olsson, Karin Sahlander, Ida von Scheele and Dr. Karin Strandberg for valuable contributions. The authors also wish to thank Dr. Per Gerde for helpful discussions.

Figure legends

Figure 1. Schematic figure of the EBC condenser

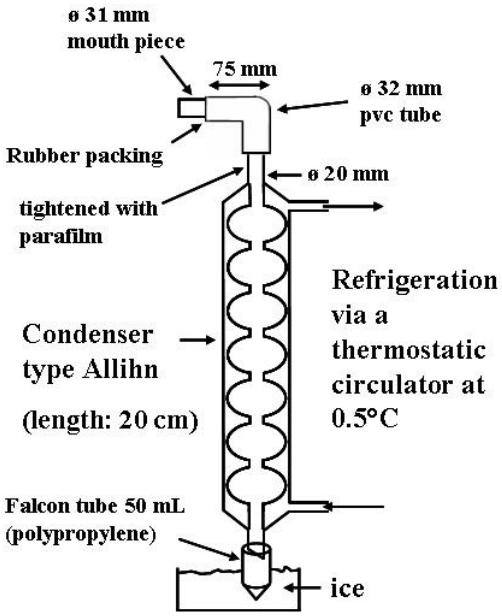


Figure 2. The figure presents the 5 exhaled breath condensate (EBC) samples (out of 102) in which leukotriene B_4 (LTB_4) or α-amylase was found to be above the detection limit. Levels of immunoreactive LTB_4 and α-amylase activity in EBC were significantly correlated (r^S=1.0, n=5).

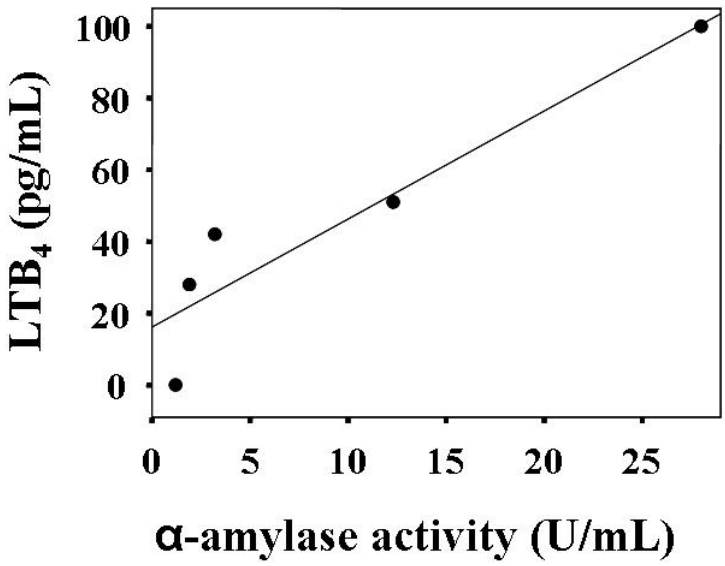


Figure 3. Levels of immunoreactive leukotriene B_4 (LTB_4) in exhaled breath condensate (EBC) and induced sputum in 11 healthy subjects before and after exposure in a swine house (n=22). The median level of immunoreactive LTB_4 in the sputum supernatants was 1190 pg/mL (193-5290 pg/mL). Immunoreactive LTB_4 could be detected only in one EBC sample collected from the same subjects up to 2 hours before sputum induction. The EBC sample had a value of 28 pg/mL, whereas the corresponding value in the sputum supernatant was 281 pg/mL.

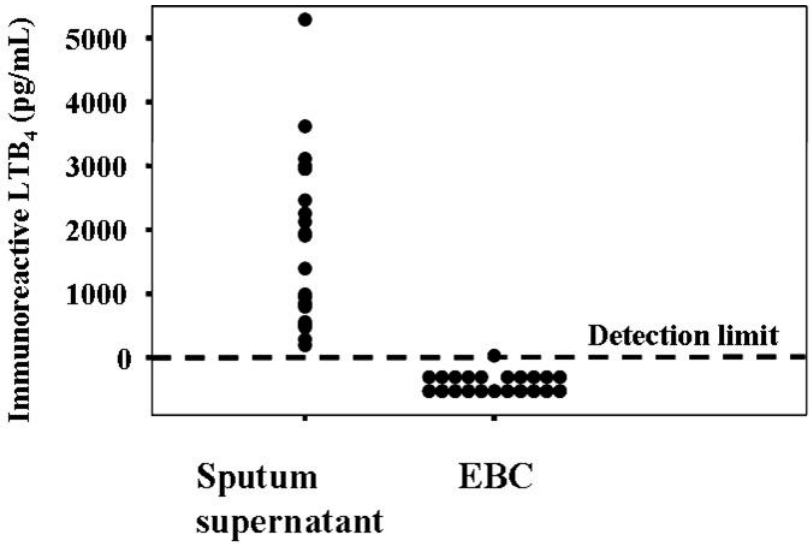


Figure 4. Stability of immunoreactive leukotriene B_4 (LTB_4) **A)** Immunoreactive LTB_4 levels were stable for 24h in room temperature in exhaled breath condensate and saliva. **B)** Immunoreactive LTB_4 levels were stable for more than 3 months when kept at -20°C.

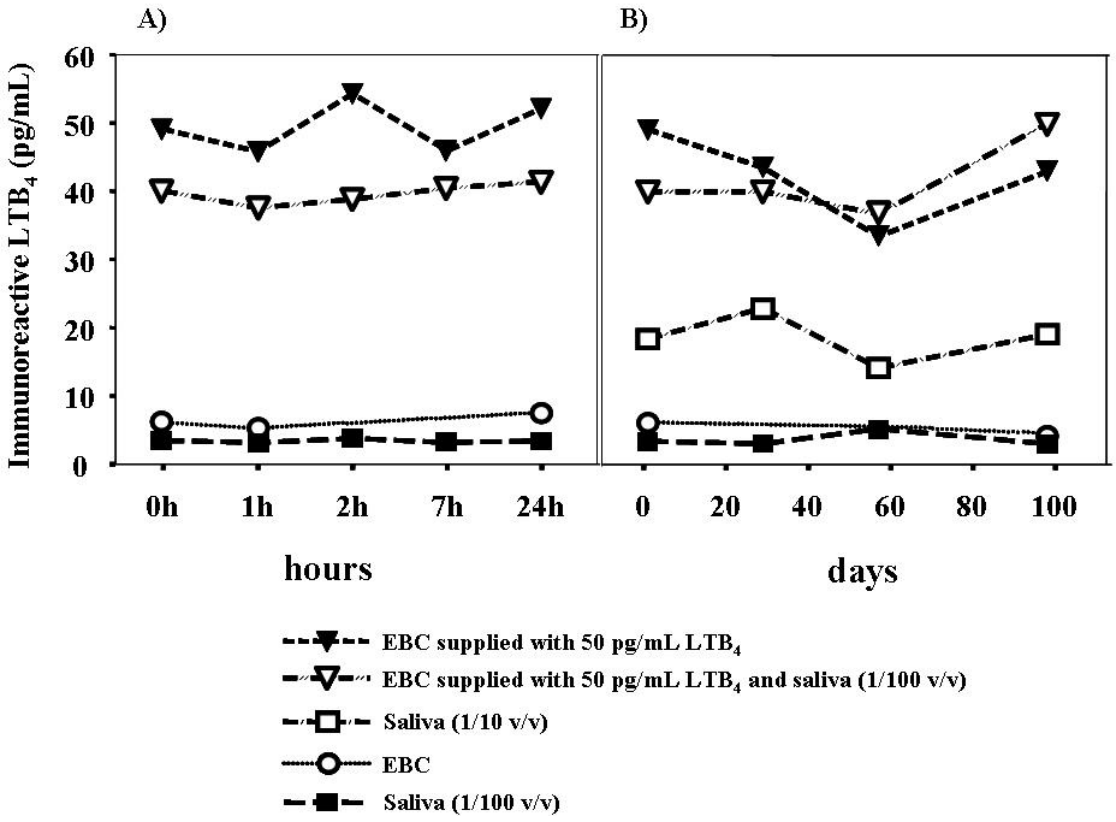
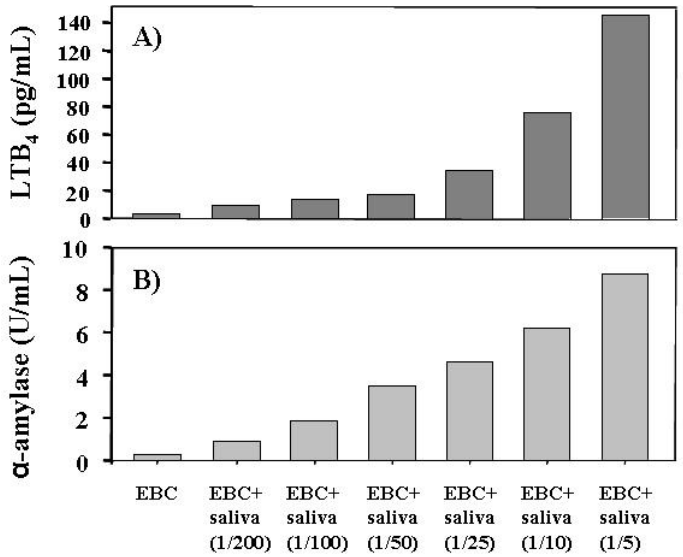


Figure 5. The figure shows increasing amounts of saliva added to EBC (v/v). **A)** Immunoreactive LTB_4, B) Alpha-amylase activity



References

1. Horvath I, Hunt J, Barnes PJ. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J 2005;26:523-48.
2. Sidorenko GI, Zborovskii EI, Levina DI. [Surface-active properties of the exhaled air condensate (a new method of studying lung function)]. Ter Arkh 1980;52:65-8.
3. Barnes PJ, Hansel TT. Prospects for new drugs for chronic obstructive pulmonary disease. Lancet 2004;364:985-96.
4. Haeggstrom JZ. Leukotriene A4 hydrolase/aminopeptidase, the gatekeeper of chemotactic leukotriene B4 biosynthesis. J Biol Chem 2004;279:50639-42.
5. Dahlen SE, Bjork J, Hedqvist P, Arfors KE, Hammarstrom S, Lindgren JA, et al. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. Proc Natl Acad Sci U S A 1981;78:3887-91.
6. Ott VL, Cambier JC, Kappler J, Marrack P, Swanson BJ. Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. Nat Immunol 2003;4:974-81.
7. Sakata K, Dahlen SE, Back M. The contractile action of leukotriene B4 in the guinea-pig lung involves a vascular component. Br J Pharmacol 2004;141:449-56.
8. Montuschi P, Barnes PJ. Exhaled leukotrienes and prostaglandins in asthma. J Allergy Clin Immunol 2002;109:615-20.
9. Carpagnano GE, Barnes PJ, Geddes DM, Hodson ME, Kharitonov SA. Increased leukotriene B4 and interleukin-6 in exhaled breath condensate in cystic fibrosis. Am J Respir Crit Care Med 2003;167:1109-12.
10. Biernacki WA, Kharitonov SA, Barnes PJ. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. Thorax 2003;58:294-8.
11. Larsson KA, Eklund AG, Hansson LO, Isaksson BM, Malmberg PO. Swine dust causes intense airways inflammation in healthy subjects. Am J Respir Crit Care Med 1994;150:973-7.
12. Larsson BM, Kumlin M, Sundblad BM, Larsson K, Dahlen SE, Palmberg L. Effects of 5-lipoxygenase inhibitor zileuton on airway responses to inhaled swine house dust in healthy subjects. Respir Med 2005.
13. O'Sullivan S, Dahlen SE, Larsson K, Larsson BM, Malmberg P, Kumlin M, et al. Exposure of healthy volunteers to swine house dust increases formation of leukotrienes, prostaglandin D2, and bronchial responsiveness to methacholine. Thorax 1998;53:1041-6.
14. Rosias PPR, Robroeks CMH, Niemarkt HJ, Hadfoune Mh, Jobsis Q, Dompeling E. The coating of the condenser systems influences the detection of 8-isoprostane in exhaled breath condensate (EBC). In: European Respiratory Society; 2004 September 4-8; Glasgow; 2004. p. 468s (Abstract).
15. in 't Veen JC, de Gouw HW, Smits HH, Sont JK, Hiemstra PS, Sterk PJ, et al. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. Eur Respir J 1996;9:2441-7.
16. Calzyme laboratories Inc. alpha-Amylase. http://www.calzyme.com/commerce/catalog/spcategory.jsp?category_id=1003. Date last updated: 2004. Date last accessed: November 1, 2005.

17. Kostikas K, Gaga M, Papatheodorou G, Karamanis T, Orphanidou D, Loukides S. Leukotriene B4 in exhaled breath condensate and sputum supernatant in patients with COPD and asthma. Chest 2005;127:1553-9.
18. Montuschi P, Martello S, Felli M, Mondino C, Barnes PJ, Chiarotti M. Liquid chromatography/mass spectrometry analysis of exhaled leukotriene B4 in asthmatic children. Respir Res 2005;6:119.
19. Pedersen AM, Bardow A, Jensen SB, Nauntofte B. Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis 2002;8:117-29.
20. Montuschi P, Macagno F, Parente P, Valente S, Lauriola L, Ciappi G, et al. Effects of cyclo-oxygenase inhibition on exhaled eicosanoids in patients with COPD. Thorax. 2005;60:827-33.
21. Salimetrics LLC. Salivary alpha-amylase assay kit. http://www.salimetrics.com/amylasekitinsert.htm. Date last updated: 2005. Date last accessed: November 22, 2005.
22. Larsson BM, Palmberg L, Malmberg PO, Larsson K. Effect of exposure to swine dust on levels of IL-8 in airway lavage fluid. Thorax 1997;52:638-42.
23. Miadonna A, Tedeschi A, Leggieri E, Lorini M, Folco G, Sala A, et al. Behavior and clinical relevance of histamine and leukotrienes C4 and B4 in grass pollen-induced rhinitis. Am Rev Respir Dis 1987;136:357-62.
24. Cap P, Pehal F, Chladek J, Maly M. Analysis of exhaled leukotrienes in nonasthmatic adult patients with seasonal allergic rhinitis. Allergy 2005;60:171-6.
25. Marteus H, Tornberg DC, Weitzberg E, Schedin U, Alving K. Origin of nitrite and nitrate in nasal and exhaled breath condensate and relation to nitric oxide formation. Thorax 2005;60:219-25.
26. von Scheele I, Sundblad B-M, Larsson K, Palmberg L. Pig Farmers, but not smokers have an attenuated airway response to organic dust and LPS. In: European Respiratory Society; 2005 September 17-21; Copenhagen; 2005. p. 600s (Abstract).