Applied Cardiopulmonary Pathophysiology 5: 215–219, 1995.

Leukotriene B_4 in breathing condensate of patients with bronchopulmonary diseases and of normal patients

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Accepted 3 July 1995

Key words: lower airways, inflammatory mediators, breathing condensate, bronchial asthma, airway diseases, diagnostics, methodology

Summary

The aim of the study was to validate a method for collection of non-volatile substances from the exhalation. These exhaled non-volatile substances were collected in a special breathing condensate (BCO) set-up, patent pending. We developed equipment used to prevent any altering effect on the biochemical properties of the specimens. The method was tested on healthy volunteers and patients with inflammatory airway diseases e.g. asthmatics. Especially mediators released by inflammatory cells into the airways were detected and quantified in exhalation.

The amount of exhaled LTB4 strongly correlated with the clinical stage of bronchial asthma, i.e. the degree of airway inflammation. In contrast, no correlation was found between exhaled LTB_4 and FEV_1. Other mediators, proteins, peptides, amino acids, phospholipids and smaller molecules were detectable in breathing condensate in previous pilot tests.

BCO collection and biochemical analysis is well-suited for use as a diagnostic tool in inflammatory airway conditions.

Abbreviations: LTB_4 – leukotriene B4, BAL – bronchoalveolar lavage, BALF – bronchoalveolar lavage fluid, BCO – breathing condensate, FEV_1 – forced expiratory volume in 1 second, RIA – radioimmunoassay

Introduction

In view of the importance of diagnostic insight into ongoing inflammatory processes in the airways, in bronchial asthma cases it is helpful to identify and measure typical parameters of inflammation, recovered as closely as possible to the site of origin and as immediately as accessible. After their release into the bronchi it is well known from *in-vitro* studies and from human studies using BAL and bronchoscopy that a great number of mediators are released into the airways by activated inflammatory cells. Since the expired air is not the mere product of alveolar gas exchange and of airway water loss, it most likely contains metabolic products stemming from ongoing inflammatory reactions in the mucosa, we addressed ourselves to the detection of mediators in the exhaled air condensate, thus avoiding their assessment in BAL fluid or in induced sputum. In a preliminary study designed to substantiate this thesis we proved that a representative mediator of mucosal inflammation, LTB_4, is exhaled and can be measured in breathing condensate [5]. It was proved that exhaled air also contains significant amounts of proteins, IL-1β, soluble IL-2 receptor proteins and TNF-α [4] as well as hydrogen peroxide [1]. The amounts of substances described varied greatly and have to be validated by well-described sampling methods.

Methods

For collection of BCO original equipment for laboratory use was developed. A patent has been applied for by the FILT Research Soc. Ltd. for the technical principles for the collection of BCO.

Breathing condensate for this pilot study was collected in a special plastic U-sized tube of 60 cm length and 20 mm diameter fitted in a freezing container filled with dry ice. The container was closed and isolated. Both outlets of the plastic tube were placed outside of the container. Volunteers were connected to the system by removable mouth piece via a non-rebreathing valve.

The sealed tubes were kept frozen at - 70° C until preparation. For measurement of LTB_4 content the samples were thawed and weighed before freeze-drying at - 10° C. The material then was resolved in 0.3 cc phosphate buffer.

The measurement of LTB_4 content was done using B_4 (3 H) RIA Kit (Advanced Magnetic Inc., Cambridge, Mass. 02138). The LTB_4-Kit used is highly specific for LTB_4. Cross reactivity for further leukotrienes, HETE, prostaglandins and thromboxanes is below 1% in practice. By that reason it was not necessary to prove LTB_4 measurement by another reference method such as HPLC.

For analysis the samples were incubated with a 3H-marked LTB_4-antibody. Antibody-bounded material was centrifuged for separation of non-bounded antibodies. The activity of remaining non-bounded antibodies was measured by Fluid Scintillation Counter Beckman LS-5000 TD. For enhancement scintillation fluid 'Ready-Safe' of Beckman was used.

A standard curve with known rates of LTB_4 concentration was taken for calibration of the counter. The counting rate was fitted for a standard between 550 and 3000 counts, i.e. 4,1–1000 pg/ml LTB_4-concentration. In case of a counting rate outside of this range the specimen was analysed again, dissolved in higher amounts of phosphate buffer to achieve a counting rate within the fitted range, later corrected for the factor of dilution. The measured LTB_4 concentration was calculated for the initial volume of each sample for standardization of the concentration in BCO.

A protocol was prepared for sampling BCO, keeping the material as intact as possible. Volunteers had to perform 15 minutes of breathing at rest via the non-rebreathing valve connected with the plastic tube.

For evaluation of the BCO sampling method healthy volunteers and patients of a pulmological outpatient department were asked to take part in a pilot study. The study was designed according to the Helsinki Declaration revised in 1983 and the ethical standards of the regional committee were fulfilled.

The study included patients with the diagnoses bronchial asthma, dry cough without bronchial hyperresponsiveness, chronic bronchitis (symptoms for more than three months per year over more than two years), seasonal allergic rhinitis without bronchial symptoms during the season and healthy controls with no hint for airway diseases in their case history.

On patients with bronchial asthma a FEV_1 was taken and the clinical staging of the disease proceeded according to Int. Consensus Report [2]. Patients with only one sample of BCO were in a stable clinical stage for more than three weeks. In one patient with unstable asthmatic disease a BCO was taken 6 times.

Clinical evaluation followed the staging of asthma according to the Int. Consensus Report [2] (Table 1).

Results

One hundred and seven volunteers were included into the study, aged between 20 and 60 years. Among them were 93 patients and 14 healthy controls. The patients were differentiated into asthmatics at various stages of the disease (51 cases), patients with chronic bronchitis (28 cases), patients with dry cough without bronchial hyper responsiveness (6 cases) and patients with seasonal allergic rhinitis without any bronchial symptoms (8 cases) (see also Table 2). In one patient with highly unstable asthmatic disease the collection of BCO was repeated six times.

Frozen breathing condensate in the plastic tube was shaped as small globules. In contrast freezing water from ultrasonic nebulizer formed crystals. Breathing condensate also seems to contain surface active substances like surfactant phospholipides and glycoproteins, not measured in this study because of the small amount of specimens.

In all samples LTB_4 was detected in different concentrations. The lowest mean levels were found in healthy controls and in patients with allergic rhinitis. In all patients with bronchial diseases higher amounts of LTB_4 were recovered in breathing condensate (Table 2). In the one patient with highly unstable asthma extremely different LTB_4 levels were found strongly correlating with the severity of symptoms and the therapeutic success (Fig. 1).

There at least appears to be a good quantitative correlation between LTB_4 exhaled and airway inflammation (polynomial regression $y = 399{,}8 - 937{,}8 \times + 615{,}5 \times^2$; $R = 0{,}45$). Yet, this observation was not reflected in the relation between LTB_4 and lung func-

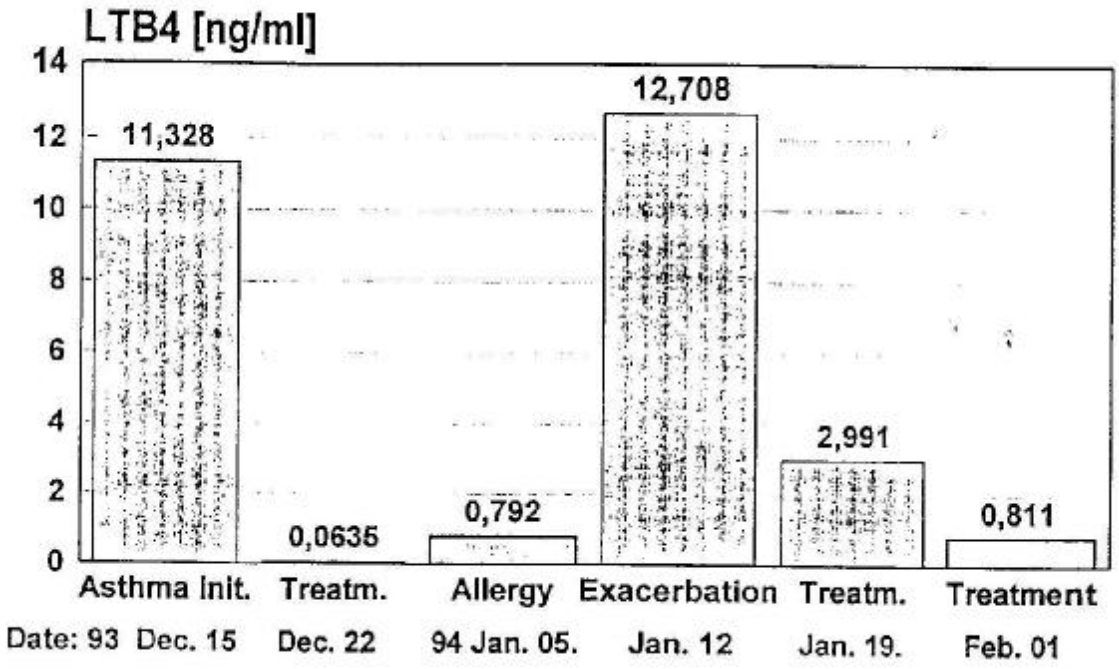

Fig. 1. Individual LTB_4 levels in breathing condensate of one asthmatic patient according to different phases of symptoms and therapeutic effectivity [LTB_4 in ng/ml]. Asthma init.: initial value at first clinical visit (asthma stage III). Treatm.: β2-mimetic daily, inhalative corticoids (stage I). Allergy: onset of desensitisation to grass pollen (stage I). Exacerb.: exacerbation of asthmatic symptoms (stage III).

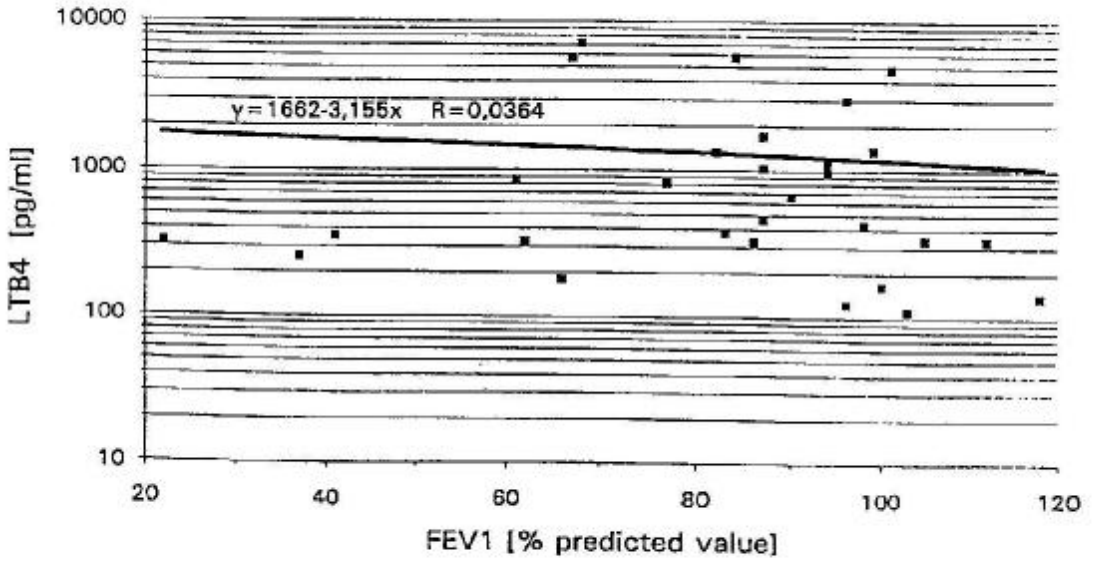

Fig. 2. Correlation between FEV_1 [% predicted value] and LTB_4-concentration (individual data of all asthmatics in stages I–III) in breathing condensate.

Table 1. Classification of asthma degree of severity according to the International Consensus Report [2].

Parameter	Asthma staging		
	Light	Medium	Severe
PEF	> 80% pred. value daily variation < 20% fully reversible	60–80% pred. value daily variation 20–30% fully reversible	< 60% pred. value daily variation > 30% not reversible
Attacks/ symptoms	< 1–2 weekly nightly < 2 per month	1–2 weekly nightly > 2 per month	very often nightly often
Treatment	β2-mimetics only occasionally	anti-inflammatory MDI daily, long acting bronchodilator daily β2-mimetics	high dose anti inflammatory MDI and long acting broachodilator daily systemic steroids

Table 2. Mean LTB_4 values with SEM in breathing condensate of 107 patients with different diagnoses and healthy controls (LTB_4 in pg/ml of breathing condensate).

	Control	Allergic rhinitis	Mild asthma (stage I)	Moderate asthma (stage II)	Severe asthma (stage III)	Dry cough	Chronic bronchitis
n	14	8	16	27	8	6	28
Mean	281.7	521.2	476.6	734.7**	4061.6**	669.7*	843.6*
SEM	47.9	83.1	167.9	214.1	1861.6	198.2	245.5
Median	240	269	258	448	492	689	414.5

* $p \leq 0.05$ and ** $p \leq 0.01$ vs. controls; analysis of variance and H-test.

tion as ascertained by FEV_1 measurements ($y = 1662 - 3.155 \times$; $R = 0{,}036$; Fig. 2).

Discussion

The equipment for collection of breathing condensate used in this study was suited for receiving sufficient BCO samples in nearly all cases. The samples were within a range between 0,3 and 1,5 cc. All volunteers, also the asthmatics in stage 3, were able to fulfill 15 minutes (i.e. about 100 exhalations) of breathing through the equipment via a non-rebreathing valve. The condensate received was between 3 to 20 μl in one exhalation at rest. In contrast Scheideler et al. [4] described total amounts of condensate between 15 to 127 μl during each vigorous exhalation at mean, using a different system of cool trap setup. No correlation was found between breathing volume and the amount of condensate in either study.

The amount of proteins and phospholipids in breathing condensate was not determined. Tracing for LTB_4 was successful in disclosing a strong correlation with the clinical severity of bronchial asthma. A nearly 9 fold concentration of LTB_4 was found in patients with stage 3-classified asthma compared with patients with stage 1-asthma classification according to the Int. Consensus Report [2]. The relevance of the LTB_4 levels was confirmed in an individual tracing of one subject whose clinical course fully matched LTB_4 levels, particularly during exacerbation of his disease, whilst LTB_4 in breathing condensate was within the range of healthy volunteers during a symptomless period on appropriate asthma medication (Fig. 1).

The amounts of leukotrienes in BCO seems to be not comparable to concentrations in bronchoalveolar lavage fluid (BALF). Bronchial lavage acts as an

provocation test itself. BALF further contains high amounts of cells capable for release and destruction of leukotrienes, also *ex-vivo*. Thus, further well designed studies for validation of breathing condensate and BALF seem to be necessary. Immediate fixation of BALF after collection similar to the freezing of BCO seems to be necessary for comparison of both methods. BCO should be taken before BALF in the same subject.

Further, the physico-chemical mechanism of exhalation of molecules and small particles is unclear. The role of surfactant material in the airways enclosing particles for clearing mechanisms should be taken into account. It seems possible to detach surfactant materials during rapid diminishing of the surface and in the course of jet effects in small airways during exhalations. Further studies should clarify the possible role of surfactant materials in the exhalation of non-volatile substances.

No correlation was found between LTB_4 and FEV_1 in percent of predicted value (Fig. 2). Thus, FEV_1 apparently is not strongly linked to the inflammatory process in the airways. This is not unexpected since LTB_4 is not necessarily a marker of contractile activity of bronchial tissue but may merely reflect a present state of inflammation as also encountered in non-spasmodic inflammatory diseases of the airways. The release of LTB_4 is a primary response of the stimulation of inflammatory cells, whereas a decrease of FEV_1 should be understood as a result of a complex regulatory response including local release of bronchoconstrictory and dilatatory mediators, local reflex mechanisms in the airways and nerval response. This conclusion however awaits further confirmation. It seems to be supported by our findings in various other inflammatory conditions of the bronchi. Yet, we also found low but significant levels of LTB_4 in symptom-free healthy controls. This may reflect permanent bronchial defence activity, probably stimulated by pathogenic organisms or by chemical pollutants or substances inhaled.

Our study was the first to prove that inflammatory mediators can be traced and measured quantitatively in condensed exhaled air. Using the method described we obtained native samples from the lung without interfering with their *in vivo*-function by invasive methods of specimen collection, e.g. BAL or induction of sputum. The breathing condensate thus attained is apt to reflect the genuine state of mediators as evading from the mucosal tissue into the airstream of the bronchial tree. This opens promising perspectives as to the use of the method for diagnostics and follow-up of clinical course and treatment of lung and airway diseases.

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