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Comparative measurement of thromboxane A_2 metabolites in exhaled breath condensate by different immunoassays

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Abstract. *Objective:* Differences between detection techniques may be partly responsible for variable mediator concentrations reported in exhaled breath condensate (EBC). We compared two types of immunoassays to estimate thromboxane A_2 (TxA_2) concentration.
Materials and methods: Thromboxane B_2 (TxB_2) levels were measured by enzyme immunoassay (EIA) and TxB_2/2,3-dinor TxB_2 by radioimmunoassay (RIA) in 10 healthy subjects and 13 asthmatic patients. 2,3-Dinor TxB_2 was also determined by a separate EIA.
Results: Thromboxane was detected in all samples by RIA, but only in about 75 % of samples by EIA. 2,3-Dinor TxB_2 was detected in most samples. There was no agreement between the results of the different immunoassays. As compared to healthy subjects, exhaled breath condensates of asthmatic patients contained significantly more immunoreactivity by RIA and TxB_2 EIA (but not by 2,3-dinor TxB_2 EIA).
Conclusion: RIA and EIA resulted in vastly different absolute values. The difference found between healthy volunteers and asthmatic patients however, suggests an increased level of TxA_2 in the airways of asthmatics.

Key words: thromboxane A_2/ B_2 – Exhaled breath condensate – Radioimmunoassay – Enzyme immunoassay – Asthma

Abbreviations
BAL: bronchoalveolar lavage
COX: cyclo-oxygenase
cysLTs: cysteinyl leukotrienes
EBC: exhaled breath condensate
EIA: enzyme immunoassay
FENO: exhaled nitric oxide
FEV_1: forced expiratory volume in 1 second
LTB_4: leukotriene B_4
LTE_4:leukotriene E_4
RIA: radioimmunoassay
TxA_2: thromboxane A_2
TxB_2: thromboxane B_2
2,3-dinor TxB_2: 2,3-dinor thromboxane B_2

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Introduction

Thromboxane A_2 (TxA_2) is one of the cyclooxygenase (COX) metabolites of arachidonic acid, which has been implicated in inflammatory processes that occur in asthmatic airways [1]. Since TxA_2 is rapidly converted to TxB_2, a chemically more stable but biologically inactive hydration product, thromboxane synthesis most frequently is monitored by measuring TxB_2 levels.

Bronchoalveolar lavage (BAL), sputum, plasma or urine is commonly used as source to investigate the role of various eicosanoids, such as TxB_2, in diseases associated with airway inflammation. TxB_2 has been detected in induced sputum of asthmatic patients using gas chromatography-negative ion chemical ionisation-mass spectroscopy [2].

Recently, exhaled breath condensate (EBC) collection has been proposed as a non-invasive method for investigating various mediators of inflammation directly from the airways. However, due to the lack of standardized sampling procedures and/or analytical methods to measure mediator levels in EBC, care needs to be taken when comparing results from different laboratories [3]. Regarding TxB_2 measurements, Montuschi et al. [4] have found that TxB_2 was undetectable in 20 of 27 EBC samples by a specific enzyme immunoassay (EIA) method. In contrast, TxB_2 immunoreactivity was detected in almost all EBC samples by a radioimmunoassay (RIA) method used in our laboratory [5, 6]. The apparent disagreement between these results may be the consequence of the different specificity of RIA and EIA. The EIA used by Montuschi et al. cross reacts with TxB_3 and 2,3-dinor TxB_2 (an oxidative metabolite of TxB_2) as well. On the other hand, the RIA does not differentiate between TxB_2 and 2,3-dinor TXB_2, but does not react with TxB_3. It is therefore reasonable to consider that the reason behind the higher concentrations found by RIA is the presence of 2,3-dinor TxB_2 in the samples. This metabolite has not yet been studied in the lung or in the airway lining fluid.

In the present study we aimed to compare the above mentioned two assays (EIA and RIA) using EBC samples from healthy subjects and asthmatic patients. Furthermore, we also aimed to determine the presence of 2,3-dinor TxB_2

in EBC samples . To exclude problems associated with collection and/or storage of samples, the TxB_2 level in a given EBC sample was determined in parallel by RIA and EIA. To compare the reliability of TxB_2 measurements by RIA/EIA, reproducibility studies were also performed.

Material and Methods

Study design

For each subject, EBC sample collection was followed by the determination of exhaled nitric oxide (FeNO) concentration and forced expiratory volume in 1 second (FEV_1). To compare the reproducibility of measurements, repeat samples were collected from healthy subjects within one week.

In the first part of the study (Study 1), the mediator level in a given EBC sample collected from each individual was determined in parallel by TxB_2 EIA and TxB_2/2,3-dinor TxB_2 RIA. To avoid possible changes in TxB_2 level that could occur during storage at room temperature, both kits were processed at the same time.

In the second part of the study (Study 2), EBC samples were collected from a new group of healthy volunteers and asthmatic patients to measure exclusively 2,3-dinor TxB_2 using another EIA.

Study 1

A total of 10 healthy volunteers and 13 patients with bronchial asthma of variable severity were studied (Table 1). All subjects were non-smokers. Healthy volunteers were non-atopic, with no history of any chronic diseases. All patients met the American Thoracic Society diagnostic criteria for bronchial asthma. Nine patients were diagnosed with atopy and one with aspirin sensitive asthma. Three patients were treated with inhaled β_2-agonists only and ten patients required inhaled corticosteroids.

Study 2

A total of 12 healthy volunteers and 17 patients with bronchial asthma of variable severity participated in the second part of the study (Table 2). Eight patients were diagnosed with atopy and one with aspirin sensitive asthma. Three patients were treated with inhaled β_2-agonists only and fourteen patients required inhaled corticosteroids.

All patients participating in the study were instructed to refrain from the use of bronchodilators for at least 8 h before the study. The protocol was approved by the local ethics committee, and written informed consent was obtained from each subject before the study.

Lung function test and exhaled NO (FENO) measurement

FEV_1 was measured using an electronic spirometer (MEDICOR MS-11, Budapest, Hungary). FEV_1 values were expressed as a percentage of the predicted normal value for the subjects height and age, according to European Community for Coal and Steel reference values for FEV_1. The best of 3 consecutive manoeuvres was accepted for evaluation.

Exhaled NO was measured by a chemiluminescence analyser (Model LR2000^R, Logan Research, UK), sensitive to NO from 1 to 5000 ppb by volume, and with a resolution of 0.3 ppb [7].

Sample collection

EBC samples were collected using an EcoScreen® condenser (Jaeger, Hoechberg, Germany) by a previously published method [8]. Subjects

Table 1. Characteristics of patients participating in Study 1

Patients sex	atopy	Age, years	Basline FEV_1%	FeNO, ppb	Steroid µg/day
F	+	24	91	6.2	250 F
M	+	39	97	7.3	0
F	+	25	94	3.4	250 F
F	+	25	95	8.4	400 B
F	+	25	91	6.2	250 F
F	+	55	86	6.4	500 F
F	+	52	93	7.1	800 B
M	+	21	88	16.1	800 B
F	+	30	79	8.7	800 B
F	–	29	86	2.5	0
F*	–	52	106	27.9	500 F
F	–	29	90	3.5	0
F	–	40	73	4.5	1200 B
Median (min-max)		29 (21–55)	91 (73–106)	6.4 (2.5–27.9)	

F/M: female/male; *aspirin sensitive patient; FeNO: exhaled nitric oxide, ppb; Baseline FEV_1%: the highest of three pre-exercise measurements of FEV1, values as % of predicted; F: inhaled fluticasone; B: inhaled budenoside

Table 2. Characteristics of patients participating in Study 2

Patients sex	atopy	Age, years	Basline FEV_1%	FeNO, ppb	Steroid µg/day
F	+	18	113	16.5	0
F	+	40	64	4.9	1600 B
F	+	28	89	11.9	250 F
M	+	34	45	7.4	500 F
M	+	35	75	19.4	0
F	+	52	84	20.6	0
F	+	26	81	10.5	600 B
F	+	31	69	4.0	400 B
M*	–	38	75	10.6	800 B
F	–	55	54	34.4	1000 F
F	–	36	92	13.9	600 B
F	–	38	85	6.0	500 F
F	–	51	129	2.5	250 F
F	–	34	88	5.0	250 F
F	–	67	100	9.2	400 B
F	–	65	64	4,9	800 B
F	–	29	64	10.5	800 B
Median (min-max)		36 (48–67)	81 (45–129)	10.6 (2.5–34.4)	

F/M: female/male; *patient with aspirin sensitive asthma; FeNO: exhaled nitric oxide, ppb; Baseline FEV_1%: values as % of predicted; F: inhaled fluticasone; B: inhaled budenoside

were asked to breathe at a normal frequency and tidal volume, wearing a nose clip for a period of 10 min. Condensates were stored at –70 °C for no longer than 1 month before measurements.

Detection of TxB_2/2,3-dinor TxB_2 by RIA

TxB_2/2,3-dinor TxB_2 concentration was determined by a specific radioimmunoassay method with a detection limit of 13.6 pg/mL and an intra-assay coefficient of variation of 9.8-4.9-3.8 %, at 50-150-250 pg/mL, respectively (Institute of Isotops Co, Ltd. Budapest, Hungary) [5]. Other characteristics of this method are: standard concentration that causes 50 % of maximal binding: 162 ± 24.5 pg/mL for TxB_2; cross reactivity: 100 % for 2,3-dinor-TxB_2 (a β-oxidation metabolite of TxB_2), 3.4 % for 11-dehydro-thromboxane B_2 (an enzymatic metabolite of TxB_2) and 2.5 % for prostaglandin D_2. According to the manufacturer, after solid-phase extraction, the main immunoreactive peaks co-migrate with tritiated TxB_2 in plasma and 2,3-dinor TxB_2 in urine.

Detection of TxB_2 by EIA

TxB_2 concentration was measured in the same EBC samples using a commercial enzyme immunoassay kit with a detection limit of 13 pg/mL (Cayman Chemical, Ann Arbor, Michigan). According to the manufacturer, the standard concentration that causes 50 % of maximal binding is 50 pg/ml; antiserum cross-reactivity is 200 % for TxB_3 and 9.9 % for 2,3-dinor TxB_2.

Detection of 2,3-dinor TxB_2 by EIA

2,3-Dinor TxB_2 concentration was measured in EBC samples using a commercial specific enzyme immunoassay kit with a detection limit of 23 pg/mL (Cayman Chemical, Ann Arbor, Michigan). Standard concentration that causes 50 % of maximal binding is 112 pg/mL; antiserum cross-reactivity that is greater than 1 % is 38 % for thromboxane B_2.

Statistical analysis

Statistical analyses were performed by GraphPad Prism Version 3 (San Diego, CA).

Data are expressed as median with min – max values or with range. Values that fell under the detection limit were used at face value in calculations. The Bland-Altman's test [9] was used to assess agreement of immunoreactivity between TxB_2 EIA and TxB_2/2,3-dinor TxB_2 RIA, and also to assess the reproducibility of measurements by either EIA or RIA in healthy volunteers. Correlations between TxB_2 EIA and TxB_2/2,3-dinor TxB_2 RIA were calculated by the Spearman's rank method. Comparisons between the two groups of subjects were performed by the Mann-Whitney test because some data were below the detection limit. Significance was defined as $p < 0.05$.

Results

Agreement between measurements by TxB_2 EIA and TxB_2/2,3-dinor TxB_2 RIA

Immunoreactivity was above the detection limit in all samples using RIA. In contrast, the immunoreactivity by EIA did not reach the detection limit for 6 samples (all from healthy subjects). There was no agreement between immunoreactivities measured by TxB_2/2,3-dinor TxB_2 RIA and TxB_2 EIA. The mean difference with two standard deviations (mean 2SD) was 98 ± 208 pg/mL (Fig1). Nevertheless, the close correlation ($r = 0.728$, $p < 0.001$) between EIA TxB_2 and RIA TxB_2/2,3-dinor TxB_2 levels indicates that the difference between the two assays is relatively constant (Fig 1, Inset).

Reproducibility of measurement with RIA and EIA

EBC samples were obtained from healthy subjects at 9 a.m., twice in one week. Each paired sample was measured by both TxB_2/2,3-dinor TxB_2 and TxB_2 EIA. The reproducibility was different depending on the method applied. The mean of day-to-day differences [mean of (1^{st} day 2^{nd} day)] with ± 2SD was –7 ± 54 pg/mL for RIA and –6 ± 30 pg/mL for EIA (Fig.2A,B). Based on the mean of average TxB_2 levels and SD values, the intra-subject coefficient of variation (CV) was 75 % for EIA and 26 % for RIA.

2,3-Dinor TxB_2 levels measured by EIA

The 2,3-dinor TxB_2 levels were below the detection limit of EIA in 7 of the 29 samples, but were above the detection limit in the rest of the samples (Fig. 3, inset).

Comparison of TxB_2 and 2,3-dinor TxB_2 levels in asthmatic patients versus healthy subjects

Using values from either TxB_2 EIA or TxB_2/2,3-dinor TxB_2 RIA, a significant and similar difference in immunoreactivity was found between healthy subjects (n = 10) and asthmatic patients (n = 13) (Fig.3, main figure, A,B). The immunoreactivity was lower in healthy subjects than in asthmatic patients [median with range: 14(5–44) pg/mL vs. 32(22–70) pg/mL, respectively, $p < 0.001$ by EIA and 99(65–152) pg/mL vs. 145(105–225) pg/mL, respectively; $p = 0.001$ by RIA]. The concentration of 2,3-dinor TxB_2 as measured by another EIA assay was not different between healthy subjects and asthmatic patients [median with range: 30(8–74) pg/mL vs. 26(8–125) pg/mL, respectively (Fig. 3, inset).

Discussion

In this study, we compared different commercially available immunoassays (RIA and EIA) to estimate the level of thromboxane in exhaled breath condensate. Our results demonstrate that EBC contains not only TxB_2, but its oxidative metabolite, 2,3-dinor TxB_2 as well. TxB_2/2,3-dinor TxB_2 levels were above the lower detection limit of RIA in each EBC sample collected from either healthy subjects or asthmatic patients. In contrast, TxB_2 levels were under the detection limit of EIA in some samples, consistent with the results of Montuschi et al. [4], who had found that TxB_2 was undetectable in 20 of 27 EBC samples using the same enzyme immunoassay method. We did not find agreement between values obtained by parallel RIA and EIA measurements, but there was an observed strong positive correlation between

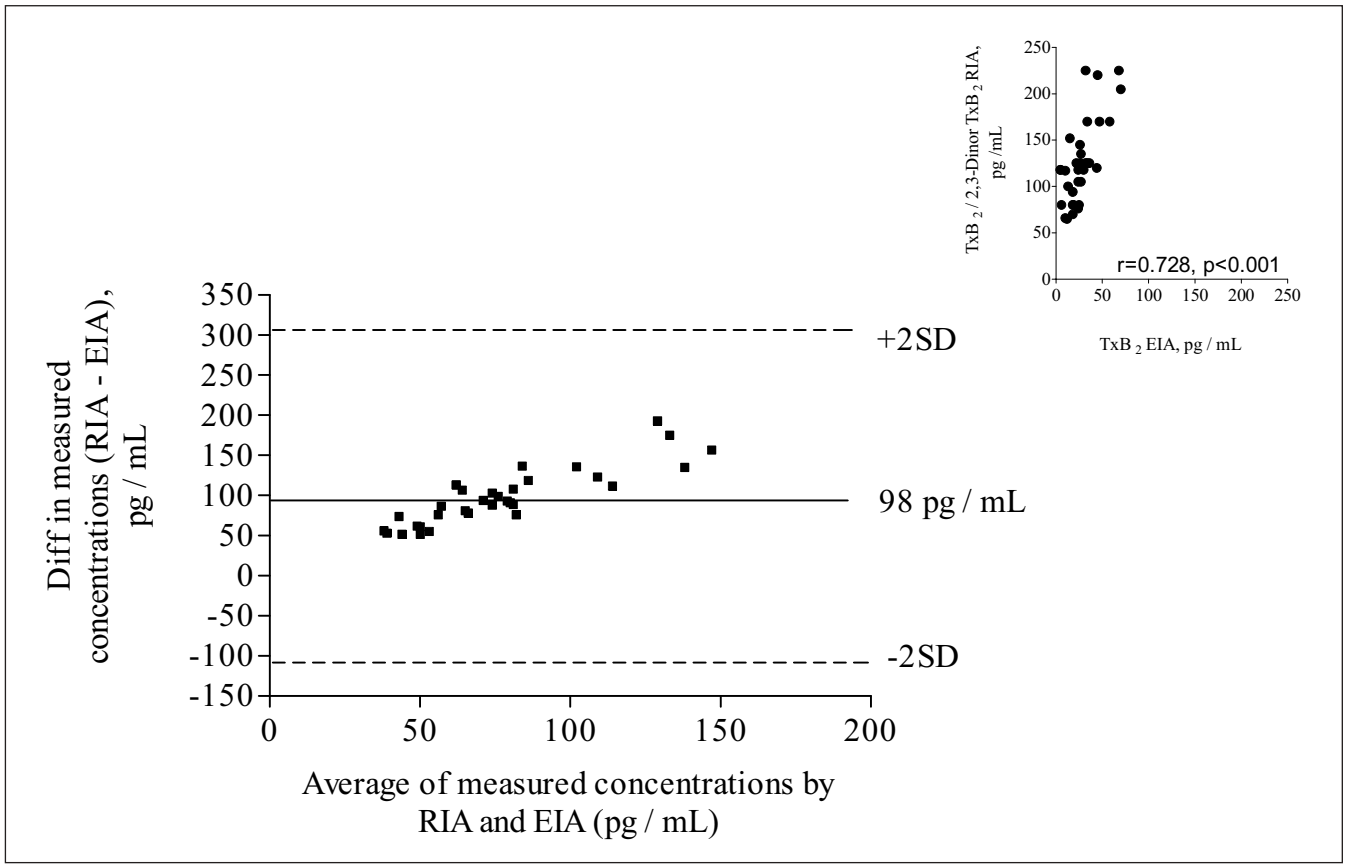

Fig. 1. Plot of differences between concentrations measured by RIA and EIA against average concentrations of exhaled TxB_2/2,3-dinorTxB_2 determined by RIA and TxB_2 determined by EIA. *Inset:* Correlation between TxB_2/2,3-dinor TxB_2 concentrations determined by RIA and TxB_2 concentrations determined by EIA. (Samples were collected on two separate occasions from 10 healthy volunteers and once from 13 asthmatic patients, n = 33)

the results indicating that the difference between the two assays was relatively constant. This suggests (although it does not prove directly) that the ratio between TxB_2 and 2,3-dinor TxB_2 is relatively stable. The reproducibility of thromboxane meausurements was good with RIA but poor with EIA. Despite the discrepancy between RIA and EIA measurements, a similar and significant difference in the levels of TxB_2 EIA and TxB_2/2,3-dinor TxB_2 RIA, but not 2,3-dinor TxB_2 alone, was found between healthy subjects and asthmatic patients.

While EIA is widely used, RIA requires a specific isotope license, therefore it is less practical and less frequently applied. Although the detection limits of the applied RIA and EIA kits are identical, their specificities are very different. The antibody used in the RIA kit does not differentiate 2,3-dinor TxB_2 from TxB_2 (100 % cross reaction), while the EIA kit is more specific (only 9.9 % specificity for 2,3-dinor-TxB_2). This is important because at the site of its production, thromboxane A_2 is rapidly hydrolyzed non-enzymatically to the relatively stable TxB_2. TxB_2 is subsequently transformed ($t^1/_2$ = 5–7 min.) to its metabolites, such as 2,3-dinor-TxB_2 by β-oxidation. There had not been an apriori reason for us to think that 2,3-dinor TxB_2 is present in the lung and we did not presume TxB_2 to undergo further extensive metabolism in our samples either. Therefore, at first, we did not measure 2,3-dinor TxB_2 by an independent immunoassay, specific for this metabolite only. Once we obtained significantly higher values by RIA, we decided to look for the presence of 2,3-dinor TxB_2 in EBC (due to technical reasons, we had to collect a new set of samples for this part of the study). Using an EIA specific for 2,3-dinor TxB_2, we found a detectable amount of this metabolite of TxB_2 in the majority of EBC samples. Due to the different cross-reactivities of the assays used, it was not possible to estimate the exact ratio of TxB_2 and 2,3-dinor-TxB_2 in our samples. We had no access to more reliable reference techniques, such as gas chromatography, to measure these metabolites. The demonstrated data indicating the presence of a relatively constant amount of 2,3-dinor TxB2 in EBC suggest that 2,3-dinor TxB_2 might, at least in part, account for the consequently higher values found by RIA vs. EIA.

Our experience shows that different batches of the RIA kit measure variable TxB_2 levels from the same EBC samples. In EBC from healthy subjects, we have previously measured 30.9 ± 19.6 pg/mL (n = 25) and 56 ± 41 pg/mL (n = 33) TxB_2/2,3-dinor TxB_2 [5, 6] and in the present study we found 99 (65–152) pg/mL (n = 10). Although the above TxB_2 levels are within the same range in all cases, the mean values and standard deviations differ highly. This observation is similar to the previously published high inter-batch variability of EIA kits that measure cysteinyl leukotrienes (cysLTs) or leukotriene B_4 (LTB_4) levels in EBC. In healthy subjects, Csoma et al. [10] found 18.5 ± 0.5 pg/mL cysLTs

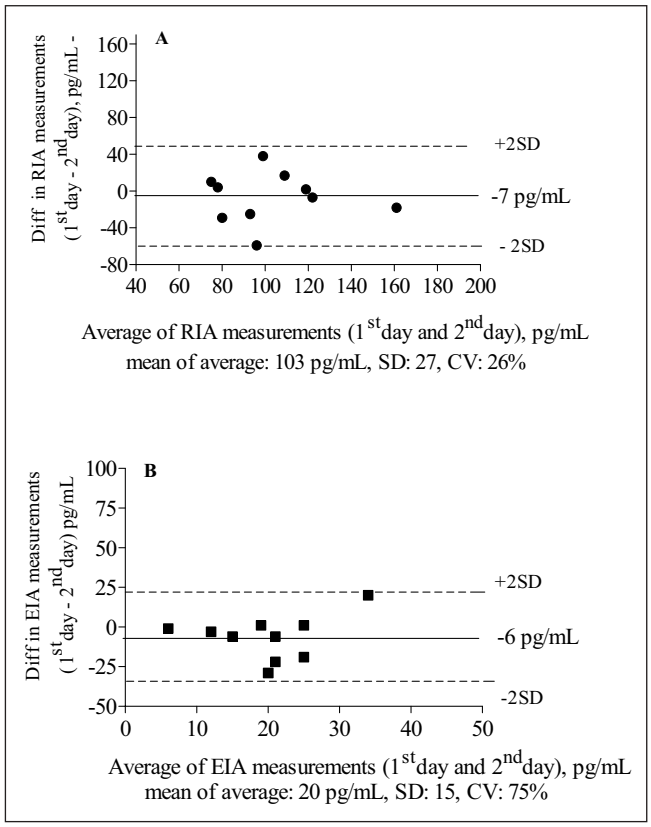

Fig. 2. Day-to-day differences in measured concentrations against mean exhaled concentrations of TxB_2/2,3-dinor TxB_2 determined by RIA (A) and TxB_2 determined by EIA (B).

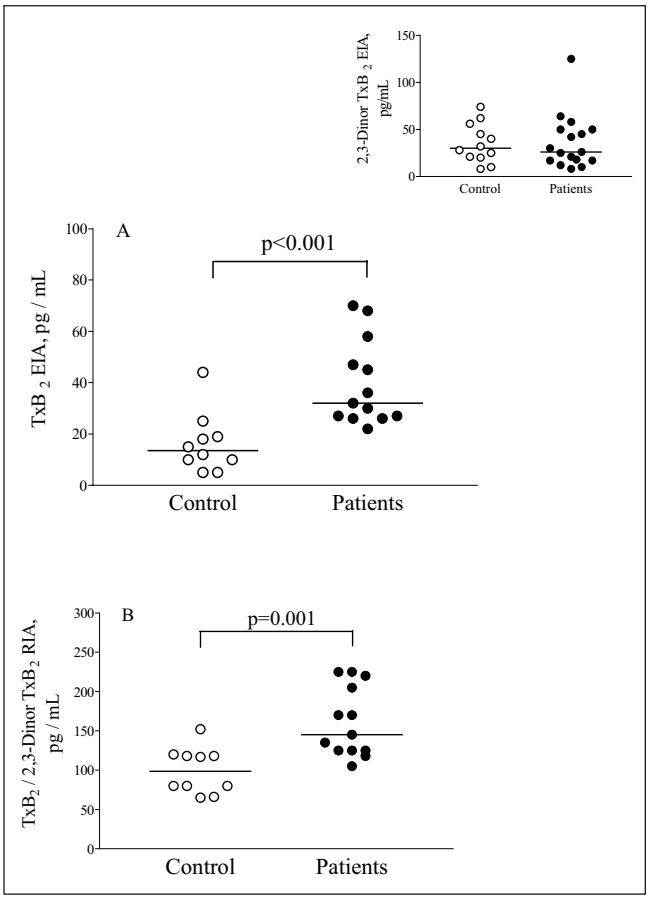

Fig. 3. Concentrations of TxB_2 determined by EIA (A) and of TxB_2/2,3-dinor TxB_2 determined by RIA (B) in EBC obtained from healthy and asthmatic patients. The difference between the two groups of subjects is significant using either method. *Inset:* 2,3-Dinor TxB_2 concentrations determined by EIA in EBC obtained from healthy and asthmatic patients.

and 47.9 ± 4.1 pg/mL LTB_4; Carpagnano et al. [11] measured 6.8 ± 0.7 pg/mL LTB_4; Pontier et al. [12] showed 76 ± 28 pg/mL cysLTs and 206 ± 18 pg/mL LTB_4 while Zanconato et al. [13] found 4.9 ± 0.8 pg/mL cysLTs. Not only in EBC, but in urine and BAL fluids of healthy volunteers as well, highly variable levels of leukotriene E_4 (LTE_4) are detected by different batches of the same EIA kit. Urinary LTE_4 concentration determined by Bochenek et al. [14] was 336.8 ± 191.1 pg/mg creatinine (n = 50) while Severien et al. [15] and Vachier et al. [16] had found 189(51–253.2) pg/mg (n = 28) and 42.5 ± 2.5 pg/mg (n = 20), respectively. The LTE_4 level in BAL was determined by Kowal-Bielecka et al. [17] to be 110 ± 67 pg/mL (n = 10), while Krawiec et al. [18] measured 31.9(27.6–69.9) pg/mL (n = 6). Not only RIA and EIA have limitations in this regard however, but the so called reference techniques as well. Gas-chromatography/mass spectrometry (GC/MS) methods – recently applied for measuring eicosanoids in EBC are also prone to give variable results. Montuschi et.al. found that LTB_4 levels in healthy volunteers were under 100 pg/mL [19] by ion-trap liquid chromatography/tandem mass spectrometry. On the other hand, Cáp et. al. found the level of this metabolite to be 205(122–369) pg/mL by GC/MS [20].

Considering the inter-batch variability of the measured values of a given metabolite, we believe that any meaningful measurement of metabolite levels by immunoassays always requires a reference point. This might be a healthy control group or, alternatively, a baseline value in prospective studies. To be able to compare results from different studies, all values need to be given as a percentage of control or baseline values. One may argue that the inter-assay variability may also be due to inter-individual variability between subjects, however this is not likely for two reasons: 1) in our experience there is a shift in the concentration of the same metabolite in EBC when assayed by different batches of the same kit, e.g. TXB_2 results from healthy subjects as published by Vass et al (5) compared with our current data: 75 % of the healthy subjects included were the same; 2) in a group of healthy subjects visiting the same laboratory for different studies there is no known explanation for the fact that one study produces an approximately 7-fold higher EBC concentration of the same mediator than the other with approximately the same standard deviation (10,11).

Our findings on elevated levels of thromboxane in asthmatics requires further study because of the low number of patients with variable severity and/or sub-type of asthma included in the present study. Also, our healthy and asthmatic groups were not precisely age-matched, although there are no data suggesting age-dependence of eicosanoids in EBC.

We demonstrated the presence of 2,3-dinor TxB_2 in EBC, therefore, it would be useful to measure not only TxB_2 but its metabolite(s) as well, to better estimate the concentration of the active molecule, TxA_2.

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