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Review

Analytical methods for the measurement of leukotrienes and other eicosanoids in biological samples from asthmatic subjects

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Abstract

This paper summarizes methodological aspects of measurements of eicosanoids in biological samples and describes some applications of such methods in studies on leukotriene formation in the human airways and the effects of drugs interfering with these compounds in asthmatic subjects. For estimations of in vivo production of eicosanoids, major, stable metabolites were selected for analysis in biological fluids. An enzyme immunoassay for LTE_4 was validated for use in unextracted urine samples. To monitor thromboxane production, a radioimmunoassay for 11-dehydro-TXB_2 was developed and used for measurements in samples of human plasma and urine. In vitro production of leukotrienes in chopped human lung was measured by UV-spectroscopy after extraction and separation on RP–HPLC. Corrections for losses during purification were performed with individually selected internal standards.

Keywords: Reviews; Eicosanoids; Leukotrienes; Thromboxanes

Contents

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1. Introduction

Eicosanoids, e.g. prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) are all biologically active oxygenated metabolites of arachidonic acid with slightly different physicochemical properties [1,2]. Prostaglandins and thromboxanes are formed via prostaglandin H synthase (PGHS) catalyzed reactions, whereas the initial enzyme in the biosynthesis of leukotrienes is 5-lipoxygenase (5-LO) (Fig. 1). Measurements of eicosanoids in tissues, biological fluids, and cell cultures have been used to establish their biological roles in a number of diseases and pathological conditions, such as allergic and inflammatory disorders [3].

The biological actions of LTC_4, LTD_4, and LTE_4, collectively referred to as the cysteinyl-leukotrienes (cys-LTs), support their putative role as mediators of bronchial asthma [4]. Thus, they all provoke plasma exudation, constriction of bronchial smooth muscle, and increased mucus secretion in the airways [4].

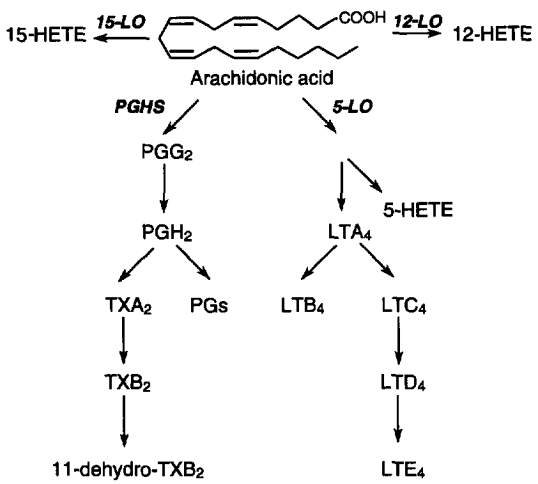

Fig. 1. Schematic overview of arachidonic acid metabolism. Prostaglandins (PGs) and thromboxanes (TXs) are formed via cyclooxygenase (PGHS) catalyzed reactions and hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs) via lipoxygenase catalyzed reactions.

Cysteinyl-leukotrienes are formed in human lung cells in vitro [5] and enhanced release was demonstrated after allergen challenge of lung specimens from asthmatic subjects [6]. More recently, in vivo formation of cysteinyl-leukotrienes has been documented in association with allergen-induced airway obstruction [7,8]. A number of anti-leukotriene drugs, which either block the formation (biosynthesis inhibitors) or the biological actions (receptor antagonists) of the cys-LTs, have proved beneficial in terms of attenuating provocation-induced asthmatic reactions [9–11]. In order to evaluate the effects of such new putative anti-asthmatic drugs and to correlate their effects with release and action of leukotrienes, there is a great need for safe and reliable analytical methods for determination of leukotrienes in biological samples.

There are several factors that are important to consider with respect to collection, storage, and extraction of biological samples containing eicosanoids. A number of analytical methods, with different detection principles, can be used for measurements of eicosanoids, e.g. high-performance liquid chromatography (HPLC) [12], thin-layer chromatography (TLC) [13], bioassay [14], UV-spectroscopy [12], gas chromatography–mass spectrometry (GC–MS) [15,16], and immunoassay [17], all of which differ in specificity and sensitivity.

Monitoring circulating metabolites in whole blood or plasma is one approach to measure in vivo production, but circulating levels of the eicosanoids are generally very low and there is always a risk of artefactual ex vivo formation during blood sampling [18]. Urine, on the other hand, is a biological fluid that is easy to collect without any such significant risk. The pattern of metabolites in urine most likely reflects the whole body production. However, it is known that intact urinary PGE_2 and TXB_2 may,

under certain conditions, originate from the kidneys. Nevertheless, urine has been found quite useful for measuring whole body production of the cys-LTs [19–22].

Immunoassays based on radioactivity (RIA) or enzyme activity (EIA) are regarded as fast and sensitive methods suitable for analysis of different arachidonic acid metabolites [17]. Relevant antibodies are now commercially available for use in RIA and EIA. To monitor in vivo production of cys-LTs we have validated an enzyme immunoassay for LTE_4 and applied this assay on urine samples collected from healthy subjects and asthmatic patients before and after inhalation of provocative agents. The assay is routinely used in unextracted urine samples after comparisons with results obtained with samples purified by solid phase extraction (SPE) followed by HPLC prior to immunoassay. We have also developed a radioimmunoassay for 11-dehydro-TXB_2 to be used on plasma and urine samples.

In this paper I will review some of our results obtained with the above-mentioned analytical procedures and discuss them in perspective of data reported by others.

2. Experimental

2.1. Materials

Mouse monoclonal antibody against $LTC_4/D_4/E_4/F_4$ was from Adv. Magn. Inc., MA, USA and rabbit polyclonal antiserum against LTE_4 was from Cayman Chemical Company, Ann Arbor, MI, USA. ^{3}H-LTE_4, ^{3}H-LTC_4, ^{3}H-TXB_2, and ^{3}H-11-dehydro-TXB_2 were purchased from New England Nuclear, Boston, MA, USA or Amersham, UK. Acetylcholine esterase-linked LTE_4 and mouse monoclonal anti-rabbit IgG were obtained from Cayman Chemical Company. Synthetic LTB_4, LTC_4, LTD_4, LTE_4, and LTE_4 metabolites were from Cascade, UK and Cayman Chemical Company. End-capped silicic acid C_{18} columns (Chromabond®) were obtained from Macherey-Nagel, Düren, Germany. The 4-hydroxy-TEMPO free radical was from Sigma. EIA buffer (100 mg sodium azide, 23.4 g sodium chloride, 370 mg tetra sodium EDTA and 1 g BSA per liter of 0.1 *M* potassium phosphate buffer, pH 7.4) and RIA buffer (0.05 *M* Tris–HCl, pH 8) were prepared in the laboratory. A kit for analysis of creatinine was purchased from Sigma.

2.2. Methods

2.2.1. Collection and preparation of biological samples

Macroscopically normal human lung tissue, obtained from patients undergoing surgery due to lung carcinoma, was chopped with fine scissors and suspended in phosphate-buffered saline (pH 7.4; 1 g per 3 ml of buffer) [23]. In vitro incubations were performed at 37°C in the presence of different stimuli for eicosanoid production and further extractions of the samples were carried out as described [23]. Lipoxygenase metabolites of arachidonic acid were separated and quantitated by reverse-phase high-performance liquid chromatography (RP-HPLC) on C_{18} stationary phases with mixtures of methanol and/or acetonitrile with water as mobile phases. Prostaglandin B_1 [24], ^{3}H-LTC_4 [23] and 16-hydroxy-heneicosatrienoic acid (16-HHnTrE) [25] were used as internal standards for quantification of LTB_4, cys-LTs, and mono-hydroxyeicosatetraenoic acids (mono-HETEs), respectively.

Human plasma samples for thromboxane analysis were collected from the cubital vein into heparinized tubes supplied with ethanolic indomethacin (final conc. of indomethacin in the samples was 25 m*M*) [26].

Human urine samples were collected from subjects with mild atopic asthma during bronchial provocations with specific allergen and from aspirin-intolerant asthmatics in association with inhalation of lysine–aspirin, essentially following protocols published elsewhere [27–29]. The patients inhaled increasing doses of allergen or lysine–aspirin in a cumulative fashion or a single dose of allergen until forced expiratory volume in one second (FEV_1) had decreased by 20% or more. The first urine sample was collected around 07:00 h, approx. 2 h before the beginning of provocation, and urine was then collected hourly until about 7 h after the inhalation challenges. The total volumes of the samples were measured and 50-ml aliquots were immediately frozen and subsequently stored at −20°C.

Creatinine was measured in all urine samples with

a commercially available kit based on a colorimetric method using alkaline picrate. The results are expressed as ng LTE_4 per mmol of creatinine in each urine sample.

2.2.2. Purification of urine samples

Extraction and separation of leukotriene metabolites in urine samples prior to immunoassay were performed using the following protocol. Urine (4 ml) was acidified to pH 4 with formic acid, subsequently diluted with an equal volume of methanol, and supplemented with ^{3}H-LTC_4 (for RIA determinations) or ^{3}H-LTE_4 (for EIA determinations) as internal standards. Samples were left at −20°C for 1 h followed by centrifugation (400 × *g*, 5 min). Supernatants were subjected to solid-phase extraction (SPE) on activated Chromabond® C_{18} silicic acid columns. The SPE columns were washed with water (2 ml), 50% methanol (2 ml), and leukotrienes were finally eluted with pure methanol (2 ml). Methanol eluates were taken to dryness under a stream of nitrogen and residues were reconstituted in HPLC mobile phase (72:28:0.1; methanol/water/acetic acid, pH adjusted to 4.2 in the water phase, giving a final apparent pH of 5.6). Samples were injected onto a RP-HPLC column (Nucleosil C_{18}, 5 μm, 4.6 mm × 250 mm, Macherey-Nagel, Düren, Germany) eluted with mobile phase at a flow rate of 0.8 ml/min. The retention times for the different leukotrienes were determined with authentic standards and UV detection at 280 nm (Fig. 2). Four HPLC-fractions (1 min/fraction), centered around the retention time of the LTE_4 standard, were assayed with RIA or EIA. For RIA determinations, when ^{3}H-LTE_4 was used as tracer, the losses of LTE_4 could not be estimated with addition of tritiated LTE_4 since this would interfere in the final assay. Instead, ^{3}H-LTC_4 was added. This LT eluted in different fractions on RP-HPLC (see Fig. 2). The recovery of ^{3}H-LTC_4 was measured with liquid scintillation counting and used to reflect recovery of unlabeled LTE_4. The mean recoveries of ^{3}H-LTC_4 and ^{3}H-LTE_4 were found to be the same, with approx. 50% losses, over the purification procedure [31].

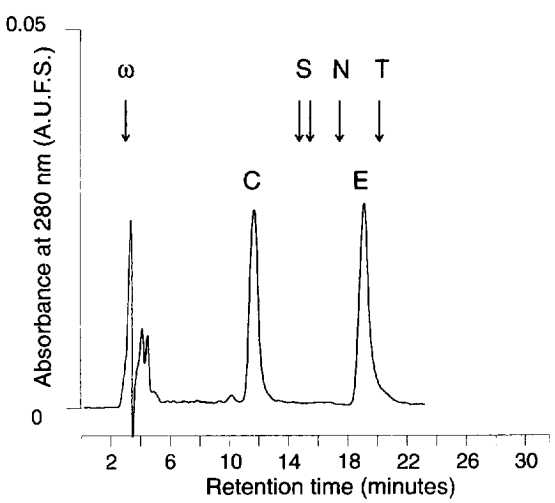

Fig. 2. RP–HPLC analysis of leukotrienes. The retention times on RP–HPLC (for details see Methods section) for synthetic leukotrienes were determined by UV-spectroscopy and are indicated with letters and arrows. ω: ω- and β-oxidized metabolites of LTE_4, all eluted with the solvent front; C: LTC_4; S: sulfon and sulfoxide deivatives of LTE_4; N: *N*-acetyl-LTE_4; E: LTE_4; T: 11-*trans*-LTE_4.

Since the EIA was sensitive to organic solvents, the four LTE_4 fractions were pooled and evaporated with a Speed Vac concentrator (Savant Instruments Inc., Farmingdale, NY, USA). The residues were resuspended in EIA buffer and assayed for content of LTE_4 with EIA. In this case recovery of LTE_4 was calculated from the recovery of ^{3}H-LTE_4 in the resuspended samples since the added radioactivity did not interfere with the EIA.

2.2.3. Stability tests

In general, no additions or adjustments of pH in the urine were performed prior to freezing of the samples. However, to determine whether the presence of antioxidant and a basic pH influences the stability of LTE_4 in the urine, separate experiments were performed. Fresh urine from a healthy non-asthmatic male subject was spiked with tracer amounts of ^{3}H-LTE_4. One sample was immediately frozen at −20°C, whereas another aliquot was supplied with the antioxidant 4-hydroxy-TEMPO free radical to a final conc. of 1 m*M* and the pH was adjusted to 9 with NaOH prior to freezing. In parallel, two samples of Tris–HCl buffer (pH 8) were supplied with ^{3}H-LTE_4, one of which was also prepared with 4-hydroxy-TEMPO and pH adjusted to 9. At days 1, 8, 30, and 60, 2-ml aliquots were withdrawn from each sample and purified as described above. RP-HPLC fractions were collected

into scintillation vials and the distribution of tritium was determined with liquid scintillation counting. Remaining intact LTE_4 over time was calculated from the amount of tritium in fractions containing LTE_4 and was expressed in percent of the amount of tritium eluting as LTE_4 at day 1.

2.2.4. Radioimmunoassay (RIA) procedure

Radioimmunoassay analysis of TXB_2 and 11-dehydro-TXB_2 was routinely performed using rabbit polyclonal antisera developed in our laboratory [26,30]. The methods were carried out as competitive assays with the use of ^{3}H-TXB_2 or ^{3}H-11-dehydro-TXB_2 as tracer and relevant synthetic thromboxane metabolites as unlabeled ligands, as described [26]. Briefly, a mixture of 100 μl of standard or 25–300 μl of unknown samples, 200 μl of 0.5% bovine γ-globulin, 100 μl each of antibody and tracer, and finally RIA-buffer to give a total volume of 700 μl, was incubated overnight at +4°C. The antibody-bound fraction was precipitated with polyethylene glycol (700 μl) followed by centrifugation and liquid scintillation counting of free radiolabeled ligand in 1-ml aliquots of the supernatants. The standard curve ranged from 6.25 to 800 pg per tube resulting in a lower limit of detection of about 20 pg/ml.

Radioimmunoassay for cys-LTs was performed essentially as described above, utilizing a mouse monoclonal antibody against $LTC_4/D_4/E_4/F_4$, ^{3}H-LTE_4 as tracer, and synthetic LTE_4 as unlabeled competitive ligand [31].

2.2.5. Enzyme immunoassay (EIA) procedure

Enzyme immunoassay was performed as a competitive assay with rabbit polyclonal antiserum and enzyme-linked (acetylcholine esterase) LTE_4 as tracer [32]. Briefly, microtiter plates (96-wells) were coated with a mouse monoclonal antibody directed against rabbit IgG followed by saturation with BSA. A mixture of 50 μl each of standard or unknown sample in several dilutions, rabbit polyclonal antiserum against LTE_4, and acetylcholine esterase-coupled LTE_4 tracer, was incubated overnight in darkness at RT. After washing, the enzyme substrate (Ellman's reagent) was added to the wells and absorbance was measured at 414 nm. With a set of standards ranging from 0.4 to 50 pg per well and % B/B_0 from 20 to 80%, the detection limit was about 8 pg/ml.

Cross-reactivities of the antibody against LTC_4, LTD_4, and several LTE_4 derivatives, were calculated as the relative ability of the respective compound to displace 50% of enzyme-labeled LTE_4 bound to the antibody. The immunoreactivity of LTE_4 was set to 100%.

3. Results and discussion

Sensitive, specific, and simple methods for analysis of eicosanoids are needed for several applications. It is of interest to measure in vitro as well as in vivo production of the compounds in order to achieve extended knowledge about the involvement of the eicosanoids in a number of pathological disorders, e.g. asthma and inflammation. In addition, the effects of drugs interfering with eicosanoid formation or action can be investigated in experimental studies only when these compounds can be accurately measured.

3.1. In vitro formation of eicosanoids

Biosynthesis of eicosanoids can be studied in vitro by incubations of cells and tissues with stimuli for release and enzymatic conversions of arachidonic acid. Intermediates in the arachidonic acid cascade, e.g. the leukotriene epoxide LTA_4, can be added as substrate for further metabolism into LTB_4 and/or LTC_4, depending on the cell or tissue studied. Leukotriene A_4 is, however, an extremely unstable compound with a half-life in buffer at RT of about 5 s [33]. The epoxide is stabilized by albumin, leading to half-lives up to 25 min at RT. Therefore, the results of in vitro incubations with LTA_4 to which albumin has been added probably better reflect the in vivo situation. Not only LTA_4, but also other unstable intermediates in the eicosanoid cascade, such as TXA_2, prostacyclin, and 14,15-LTA_4, can be stabilized by albumin [34–36].

To isolate eicosanoids from the incubations it is often necessary to extract and purify the samples. Extractions with organic solvents, e.g. chloroform, ether or ethyl acetate, only give a lipid fraction with no further separation of different classes of fatty acid

metabolites [23]. However, the use of silicic acid open-column chromatography allows separation of compounds with slightly different polarity. For instance, the cys-LTs can be separated from other LTs and PGs by this technique [12,23].

For most of the lipoxygenase products, quantification may be performed with UV-spectroscopy [12]. The different compounds absorb UV-light at different wavelengths. The λ_{max} for the cys-LTs is 280 nm whereas for the monohydroxy acids the λ_{max} is 235 nm. To be able to correct for losses during storage and work-up of the samples, the use of internal standards is important. The properties of the internal standard should be as close as possible to those of the compounds to be quantified. For example, PGB_1 is often used as internal standard for quantification of LTB_4 [24]. However, for the recovery of LTC_4, LTD_4, and LTE_4, PGB_1 is less suitable due to considerable differences in chemical properties. Instead, we have successfully used tritiated LTC_4 as internal standard for measurements of all the cys-LTs [23]. Finally, an internal standard for the mono-HETEs was synthesized in our laboratory from a C_{21} fatty acid which was hydroxylated with soybean lipoxygenase to produce 16-HHnTrE [25].

In this context, it is important to keep in mind that stereoisomers of a particular compound are not always separated on ordinary RP-HPLC stationary phases, but may require chiral phase chromatography and special chemical derivatisations [25,37]. The biological activity and even the mode of biosynthesis may differ between (*S*) and (*R*) isomers of the same eicosanoid. For example, 15-HETE may be formed either via cyclooxygenase, lipoxygenase or monooxygenase catalyzed reactions. However, 15(*S*)-HETE is formed via 15-lipoxygenase, 15(*R*)-HETE is the product of cyclooxygenase, whereas monooxygenase-derived mono-HETEs are often racemic mixtures [38].

Furthermore, metabolites of a certain compound are not always absorbing UV-light at the same wavelength as the parent compound. This is the case for hydrogenated metabolites of LTB_4 in certain cells or tissues [37]. For instance, 10,11-dihydro-LTB_4 lacks one of the three conjugated double bonds of LTB_4 and is therefore absorbing UV-light of 235 nm instead of 270 nm. When looking for metabolites it is thus of importance to monitor absorbance at several wavelengths. In fact, this technique was critical for collecting the data by which we could show that the further metabolism of LTB_4 in the lung differed considerably from the metabolic pathway previously shown to be dominating in white blood cells [39].

Our results from in vitro incubations of chopped human lung tissue, using the above-mentioned methods, are summarized in Table 1. IgE-mediated activation with allergen or anti-IgE led to release of the cys-LTs, whereas LTB_4 was only formed in response to the calcium ionophore A23187 [23]. These results suggest that cys-LTs and LTB_4 mainly originate from mast cells and alveolar macrophages, respectively [40,41]. Finally, the formation of the 15-lipoxygenase product 15-HETE was significantly augmented in bronchi from asthmatic subjects in comparison with bronchial tissue from non-asthmatic individuals [25]. The results on in vitro eicosanoid formation in human airways is summarized in Ref. [42].

3.2. Measurements of in vivo thromboxane formation

In vivo formation of TXA_2 was mimicked by i.v. injections of TXB_2 followed by RIA analysis of TXB_2 and 11-dehydro-TXB_2 of blood-plasma samples [43]. Thromboxane B_2 was short-lived in the circulation and within 15 min after injection the levels were considerably attenuated. However, when blood started to clot in the canula the levels of measured TXB_2 rose again, as an index for TXA_2 formation [43]. This production of TXA_2 was most likely a result of activation of platelets during blood sampling, leading to ex vivo formation of TXA_2 which, in turn, is immediately hydrolyzed to the non-enzymatic product TXB_2. When a new puncture site was used in the contralateral arm, the levels of measured TXB_2 in plasma were down to basal, which confirms the hypothesis of ex vivo thromboxane formation. This potential artifact may be the reason for reports on unreasonably high levels of circulating TXB_2 [18]. The addition of indomethacin to the test tubes, which blocks the PHGS and thus diminishes the TX production during platelet activation may, at least to some extent, prevent the

contribution of TXB_2 formed ex vivo to the total amounts measured. However, if one instead measures a stable and enzymatically formed major metabolite of TXB_2, these problems can be totally avoided. 11-Dehydro-thromboxane B_2 is formed from TXB_2 through the action of a tissue bound dehydrogenase and this metabolite is more long-lasting in the circulation than TXB_2 itself [43]. Even more important, 11-dehydro-TXB_2 can never be formed ex vivo since TXB_2 will not be converted to 11-dehydro-TXB_2 in the absence of the tissue bound 11-dehydrogenase [26]. Apparently, measurement of urinary 11-dehydro-TXB_2 also reflects in vivo production of thromboxane. For instance, urinary levels of 11-dehydro-TXB_2 were increased after allergen provocation of atopic asthmatic subjects [27]. This was not seen when bronchoconstriction was induced with histamine and thus the results point to a specific allergen-induced thromboxane formation [8,27]. The exact mechanism for this event is, however, not yet established.

3.3. In vivo leukotriene formation

3.3.1. Metabolism of cys-LTs

In order to decide which metabolite to use as parameter for analysis of a particular compound it is of importance to investigate its metabolism, especially in vivo. We have shown that the pulmonary metabolism of LTC_4 in vitro results almost exclusively in the formation of LTE_4 within 30 min [23]. Studies in vivo have led to the same conclusion, namely that LTE_4 is a major end-metabolite which is excreted intact in the urine and therefore serves as a suitable target for measurements of in vivo production of cys-LTs [19,44,45].

3.3.2. Stability of LTE_4 in urine

We performed controlled experiments in which the stability of LTE_4 in buffer and urine was tested. The stability of ^{3}H-LTE_4 in urine was not improved by the presence of the often-used preservative 4-hydroxy-TEMPO and pH adjustment to 9 [31]. In fact, the relative amount of intact LTE_4 tended to decrease after 60 days in the prepared samples, with 80% of control as intact LTE_4 in the presence of preservative. In buffer, this phenomenon was even more pronounced and after 60 days only 7% of the tritium was still associated with intact LTE_4 in the presence of 4-hydroxy-TEMPO [31]. The result is in agreement with a previous study where we recovered more than 90% of LTE_4 immunoreactivity after storage of untreated urine samples for approx. 10 months at −20°C.

3.3.3. Cross-reactivities of the anti-LTE_4 antibody

For analyses of LTE_4 in human urine, we have validated different immunoassays. Evidently, one important factor is the specificity of the antibody. Structurally similar compounds may cross-react with the antibody and substances in the biological matrix may interfere unspecifically with the antigen–antibody interaction. The cross-reactivities against some structurally similar compounds, of the most frequently used LTE_4-antibody in our laboratory, are depicted in Fig. 3. The cross-reactivities against N-acetyl-LTE_4 and 11-*trans*-LTE_4 were 100 and 11%, respectively. More data on cross-reactivities of this and other antibodies have been published elsewhere [31]. The cross-reactivities against LTC_4 and LTD_4 are of less importance since these cys-LTs are not present in human urine [19,45]. The ω- and β-oxidized metabolites are breakdown products of LTE_4 that are not found in the urine until about 4 h after pulmonary production of LTC_4 [46,47]. The sulfoxide and sulfon derivatives of LTE_4 could theoretically be formed non-enzymatically during work-up procedures and therefore the cross-reactivities against these compounds may be important to take into account. The N-acetyl-LTE_4 is a major metabolite in rodents, but is not significant in human urine. Finally, the *trans*-isomers of the cys-LTs, such as 11-*trans*-LTE_4, may be formed non-enzymatically and are usually not clearly separated from the parent compound on RP-HPLC (see Fig. 2).

3.3.4. Comparison between crude and purified urine samples

Most data on levels of immunoreactive LTE_4 in the urine has so far been generated with the combination of RP-HPLC and immunoassay [21,48,49]. We have validated a simpler strategy using EIA or RIA analysis on unextracted urine samples, and compared the results with measurements of the same samples after SPE and RP-HPLC separation.

Urine samples were collected hourly during al-

Table 1
In vitro formation of eicosanoids in human airways. Fragmented fresh tissues from human lung and bronchi were incubated in vitro in the presence of different stimuli. Leukotrienes and 15-HETE were separated and quantitated by RP–HPLC and UV-detection. Results are expressed in pmol per g of tissue (mean of several experiments, $n > 3$)

Stimulus	Lung			Bronchi			Asthmatic lung			Asthmatic bronchi		
	LTB_4	LTC_4–LTE_4	15-HETE	LTB_4	LTC_4–LTE_4	15-HETE	LTB_4	LTC_4–LTE_4	15-HETE	LTB_4	LTC_4–LTE_4	15-HETE
anti-IgE	nd[a]	0.1	na[b]	nd	nd	1.0	nd	0.2	0.3	nd	nd	2.0
A23187	0.3	0.3	na	nd	nd	0.8	0.1	0.3	0.3	nd	nd	2.8
AA[c]+A23187	1.0	0.2	0.7	nd	nd	4.3	0.9	0.5	0.7	nd	nd	9.3

[a]nd, not detected.
[b]na, not analyzed.
[c]AA, arachidonic acid.
For more detailed description of the results see Refs. [23], [25] and [42].

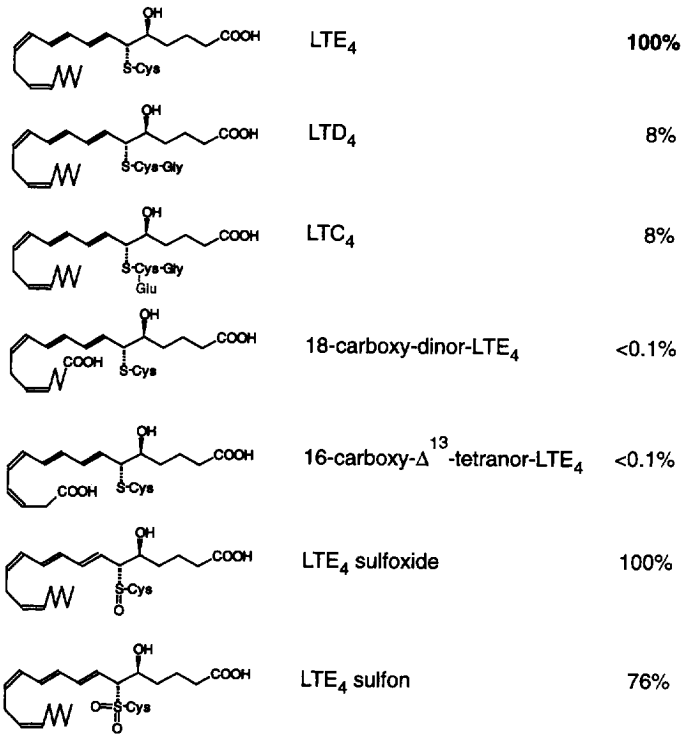

Fig. 3. Cross-reactivities of the anti-LTE_4 antibody used in EIA. The ability of some structurally similar compounds to displace the LTE_4-tracer from the antibody, measured as percent of the ability of LTE_4 itself. The compounds are enzymatically or non-enzymatically formed LT-metabolites potentially present in biological samples. For experimental details see the methods section.

lergen challenge. A comparison between results obtained with immunoassays performed on these samples before and after purification revealed no significant differences, and thus the calculated difference between baseline and post-challenge samples were the same (see also below) [28,31]. Furthermore,

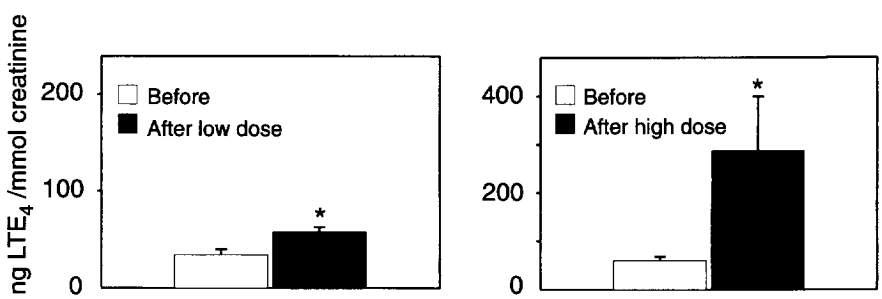

Fig. 4. Effects of bronchial provocation with allergen on urinary levels of LTE_4. Urinary LTE_4 was analyzed with RIA on unextracted samples collected during bronchial provocation with allergen. A significantly increased level of urinary LTE_4 was documented after allergen challenge. The left panel shows the results obtained with a low dose of allergen given at the placebo session. The right panel depicts the effects of a higher dose of allergen after pretreatment with a LT receptor antagonist (ICI-204,219; Accolate).

purification prior to immunoassay was disadvantageous due to considerable losses of leukotrienes (approx. 50%) during the purification as documented by tritiated internal standards [31]. Therefore, in clinical experimental studies the urine samples were routinely analyzed without prior purification. With this procedure the EIA analysis also lends itself to automization. The analyses were thus performed with a pipetting robot, MultiProbe 104 (Canberra Packard, Merideen, CT, USA).

3.4. Measurements of urinary LTE_4 after bronchial provocation with allergen

With immunoassay analyses of unextracted urine samples we have documented in vivo production and increased urinary excretion of LTE_4 after inhalation of allergen in atopic asthmatics. Simultaneously, the allergen provoked bronchoconstriction, measured as a drop in FEV_1. Urine was collected hourly and the peak excretion of LTE_4 was measured about 1 h after the maximal drop in FEV_1, with levels back to basal after about 3 h.

The effects of anti-leukotriene drugs in asthmatics were investigated in clinical experimental studies. All trials were performed following a double-blind placebo-controlled cross-over design. A significantly increased urinary excretion of LTE_4 was seen after allergen-induced bronchoconstriction in the placebo session (Fig. 4). When the patients tolerated several and higher doses of allergen after pretreatment with the LT receptor antagonist ICI-204,219 (Accolate), the increased excretion was even more pronounced (Fig. 4), most likely due to a mechanism dependent on the inhaled dose of allergen [27].

In the following experiments, bronchial provocation with a single predetermined dose of allergen was performed in the presence or absence of the LT biosynthesis inhibitor BAY X1005 [29]. We could demonstrate a significant inhibition of the allergen-induced increased LTE_4-excretion by this 5-LO inhibitor (750 mg 4 h prior to challenge; Table 2), and the allergen-provoked bronchial reaction was also inhibited to a similar degree [29,31]. The analyses of urinary LTE_4 in this study were performed both on unpurified samples and after purification by SPE and RP-HPLC on aliquots of the same samples. There was no significant difference between the values obtained on net increase in urinary LTE_4 with the two methods (Table 2).

Table 2
Levels of urinary LTE_4 after allergen challenge in the presence or absence of a leukotriene biosynthesis inhibitor with analyses performed in unpurified and purified samples. Urine samples collected hourly during allergen provocation, in the presence of placebo or LT biosynthesis inhibitor, were analysed with EIA with or without prior purification by SPE and RP-HPLC. The net increase in urinary LTE_4 excretion (post-challenge level minus pre-challenge level) was attenuated by the inhibitor. Statistical significance of differences were calculated with Student's *t*-test. Mean ± S.E.M. There was no significant difference between values obtained in unpurified and purified samples at the placebo or inhibitor sessions, respectively

	Urinary LTE_4, net increase[a] (ng/mmol creatinine)	
	Unpurified	Purified
Placebo	27±1.4	20±4.7
LT biosynthesis inhibitor[b]	7.6±3.3[c]	1.4±1.4[d]

[a]Net increase equals post-challenge level minus pre-challenge level.
[b]Bay × 1005.
[c]$P < 0.002$.
[d]$P < 0.05$.

3.5. Measurements of urinary LTE_4 after aspirin-induced bronchoconstriction

We have also studied patients with so called aspirin-induced asthma, in which aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) precipitate asthma attacks [50]. Inhalation of lysine–aspirin in these aspirin-intolerant patients, leading to a significant airway obstruction, was shown to be associated with increased excretion of urinary LTE_4. This was exclusive for the sensitive patients in that aspirin-tolerant asthmatics did not react with either bronchial obstruction or increased excretion of urinary LTE_4, in response to inhaled lysine–aspirin [27]. We could also show that the LTE_4 excretion was not a result of the bronchoconstriction per se, since the LTE_4 levels were unaltered in the urine after histamine-induced bronchoconstriction [27]. In this context, no change in the excretion of 11-dehydro-TXB_2 was seen after aspirin-induced bronchoconstriction, in contrast to the increase documented after allergen provocation (vide supra). Together with positive results from studies with anti-leukotriene

drugs [28,51,52], and elevated basal excretion of LTE_4 in aspirin-intolerant asthmatic subjects [22,27,31], the data published so far point to a very important role especially for cys-LTs in aspirin-induced asthma.

4. Conclusions

Some methodological conclusions can be drawn from the data presented above. First, it is of importance to be aware of the risk of artefactual ex vivo formation of eicosanoids, e.g. thromboxane production during blood sampling, which may lead to inaccurate estimations of in vivo biosynthesis of the respective compound. Second, extremely unstable eicosanoids can in some cases be stabilized in vitro with albumin. Third, separation of steric isomers (epimers) of eicosanoids sometimes requires specific derivatisations or chiral phase chromatography.

Moreover, we have shown that immunoassay of LTE_4 in urine is a promising way to measure in vivo production of cys-LTs and that purification of urine samples prior to immunoassay does not necessarily improve the results. We have successfully applied a simple method for measurements of LTE_4 in unpurified urine on samples collected from healthy subjects and asthmatic individuals, under a variety of experimental conditions. Using this rapid and sensitive method, we have collected a series of interesting and valuable data concerning allergen- and aspirin-induced asthma as well as effects of pharmacological intervention [27–29,31]. Finally, our method for analysis of excretion of LTE_4 in human urine may be useful in a number of clinical experimental studies where altered excretion of LTE_4 may be a part of a pathological event [53–56].

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