

Increase in urinary leukotriene B₄ glucuronide concentration in patients with aspirin-intolerant asthma after intravenous aspirin challenge

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Summary

Background Aspirin challenge of aspirin-intolerant asthma (AIA) patients causes a significant increase in leukotriene E₄ (LTE₄) concentration in urine. However, knowledge on leukotriene B₄ (LTB₄) generation in patients with AIA is insufficient. Recent research has demonstrated that exogenously administered LTB₄ is excreted as glucuronide into the urine in human healthy subjects. **Objective** The purpose of this study is to estimate urinary LTB₄ glucuronide (LTBG) concentration in the clinically stable condition in healthy subjects and asthmatic patients and to investigate changes in urinary LTBG concentration in patients with AIA after aspirin challenge.

Methods A provocation test was performed by intravenous aspirin challenge. After urine was hydrolysed by β -glucuronidase, the fraction containing LTB₄ was purified by high-performance liquid chromatography and LTB₄ concentration was quantified by enzyme immunoassay. Urinary LTBG concentration was calculated as the difference between the concentration obtained with hydrolysis and that without hydrolysis.

Results (1) After hydrolysis, the presence of urinary LTB₄ was verified by gas chromatography-mass spectrometry-selected ion monitoring. (2) The urinary LTBG concentration was significantly higher in the asthmatic patients than in the healthy subjects (median, 5.37 pg/mg creatinine [range 1.2–13] vs. 3.32 pg/mg creatinine [range, 0.14–10.5], $P = 0.0159$). (3) The patients with AIA ($n = 7$), but not those with aspirin-tolerant asthma ($n = 6$), showed significant increases in LTBG and LTE₄ excretions after aspirin challenge. (4) When the concentrations after aspirin challenge were analysed simultaneously, a significant linear correlation was observed between urinary LTBG concentration and urinary LTE₄ concentration in patients with AIA (Spearman's rank correlation test, $r = 0.817$, $P = 0.0003$).

Conclusion LTBG is present in human urine, albeit at a concentration lower than urinary LTE₄. In addition to a marked increase in cysteinyl-leukotriene production, aspirin challenge induced LTB₄ production in AIA patients.

Keywords aspirin-intolerant asthma, leukotriene B₄ glucuronide, leukotriene B₄, leukotriene E₄

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Introduction

Aspirin and related non-steroidal anti-inflammatory drug ingestion precipitates severe bronchoconstriction in a subset of patients with bronchial asthma. This syndrome is referred to as aspirin-intolerant asthma (AIA). Because these drugs inhibit cyclooxygenase, an enzyme responsible for the formation of PGs and thromboxane, it has been assumed that cyclooxygenase has a pivotal role in the initiation of the intolerance reaction, and an abnormality in arachidonic acid metabolism may be responsible in the onset of the reaction [1]. However, the mechanism underlying the intolerance reaction remains unknown. AIA patients show an increased basal production of cysteinyl-leukotrienes (cys-LTs) even under a clinically stable condition, as evidenced by an increased excretion of leukotriene E₄ (LTE₄) into the urine

[2–6]. Following aspirin administration, the patients show a further increase in urinary LTE₄ excretion [2, 7, 8]. These findings suggest that cys-LTs play a considerable role in the pathogenesis of AIA [1, 9]. Based on studies in which eicosanoids and chemical mediators were measured in biological fluids, it is most plausible that mast cells, but not eosinophils, are activated in patients with AIA after aspirin challenge [10, 11]. However, it has been impossible to determine definitely the type of cell that generates leukotriene C₄ (LTC₄) in patients with AIA after aspirin challenge despite extensive investigation.

Leukotriene B₄ (LTB₄), which is a product of another branch of the LT pathway, is a chemotactic factor for eosinophils and neutrophils [12]. LTB₄ also stimulates the adhesion of neutrophils to endothelial cells and causes degranulation and superoxide anion generation in neutrophils [13]. *In vivo* LTC₄ production has been estimated by measuring urinary LTE₄ concentration. On the other hand, the method of estimating LTB₄ production has not been established yet, because an easily accessible metabolite has

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not been identified in spite of extensive studies [14]. A recent study has demonstrated that LTB₄ glucuronide (LTBG) and 20-carboxy-LTB₄ appear in the urine after an intravenous injection of a relatively large dose of LTB₄ and that these compounds may be used as a marker for the whole-body production of LTB₄ [15, 16]. To date, there have been no studies of the measurement of these compounds in urine from humans who did not receive exogenous LTB₄. The purpose of this study was to establish a method of LTBG quantification and to measure urinary LTBG concentration in order to assess *in vivo* LTB₄ production in humans. In addition, we have measured urinary LTBG concentration after aspirin challenge in AIA patients in order to better understand the relationship between arachidonic acid metabolism and the pathogenesis of AIA.

Materials and methods

Measurement of urinary LTBG concentration

Siliconized glass tubes, polypropylene tubes and polypropylene pipettes were used throughout the study. Two urine aliquots were withdrawn from the stored urine samples, which had been collected during visits to the hospital in the morning (between 9:00 and 12:00 hours). One urine aliquot (1 mL) was subjected to enzymatic hydrolysis and the other urine aliquot (1 mL) was used for the quantification of intact urinary LTB₄ without hydrolysis. Each urine sample was loaded on an LC18 column (Supelco, Bellefonte, PA, USA). The column was washed with distilled water, and LTB₄ and its glucuronide were eluted with 3 mL of methanol. The methanol extract was concentrated under reduced pressure and dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.0). The solution was incubated with 200 U of β -glucuronidase (G7646, Sigma, St Louis, MO, USA) at 37 °C overnight. After extraction using an Empore C18 disk cartridge (3M, St Paul, MN, USA), LTB₄ was purified by high-performance liquid chromatography (HPLC). HPLC was performed on a NOVA-PAK C18 column (Waters, Milford, MA, USA) with a solvent mixture of methanol-distilled water-acetic acid (65:35:0.1, v/v/v) containing 0.1% EDTA (pH adjusted to 5.4 with ammonium hydroxide) at a flow rate of 1.0 mL/min at 37 °C [17]. LTB₄ and LTE₄ were eluted at 10.1 and 11.1 min, respectively, and two distinct peaks were obtained. The fraction with a retention time corresponding to that of LTB₄ was collected and LTB₄ was recovered from eluates with Empore C18 disk cartridge. The method using Empore C18 disk cartridge is rapid, yields reproducible results. LTB₄ concentration was determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA). The value obtained for the sample treated with β -glucuronidase was subtracted from the amount of LTB₄ to obtain the amount of LTBG. The concentrations were expressed as picogram per milligram of creatinine.

LTB₄ identification by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM)

LTB₄ was converted to its pentafluorobenzyl ester di-trimethylsilyl ether derivative as reported previously [18].

When the derivative was analysed by GC-MS in the negative-ion chemical ionization mode using methane as reagent gas, the mass spectrum of the derivative revealed two prominent ions. The ion at m/z 479 was formed as a base ion following the elimination of the pentafluorobenzyl fragment from the molecular ion, and this ion further fragmented to the ion at m/z 389 following the loss of the trimethylsilyl alcohol fragment. Due to thermal rearrangement, the retention time of the ion at m/z 389 was slightly longer than that at m/z 479 [18]. The mass spectrum was measured using Shimadzu GC-MS QP2010 (Kyoto, Japan) equipped with an SPD-5 capillary column (15 m, 0.25 mm internal diameter, 0.25 μ m film thickness; Supelco). The ion source and interface temperatures were set at 250 °C. The initial column temperature was maintained at 150 °C for 2 min and then increased to 300 °C at 10 °C/min. The retention time of the derivative was about 9.6 min under these conditions.

Measurement of recovery rates of LTB₄

When we measured the recovery rates of LTB₄ using a ³H-labelled compound, radioactivity remained even when the ³H-labelled compound was decomposed after purification by chromatography, as long as the ³H label remained intact. That is, radioactivity would not identify the ³H-labelled compound itself and it is impossible to measure the correct recovery rates of LTB. Therefore, we used the following method of measuring recovery rate. (1) We added 10 pmol each of deuterium-labelled LTB₄ (d4-LTB₄, Cayman Chemical) and LTB₄ to the urine. We then extracted them with ethyl acetate at pH 1.0 and converted them to pentafluorobenzyl di-trimethylsilyl ether derivatives. The peak areas of the derivatives were measured by GC-MS-NCI at m/z 479 and at m/z 483, which corresponded to fragment ions derived from LTB₄ and d4-LTB₄, respectively. We assumed that the ratio of the peak area of m/z 483 to that of m/z 479 is A . (2) We added 10 pmol of LTB₄ to the urine, which was then purified using all the methods, and after the purification, 10 pmol of d4-LTB₄ was added. We measured the ratio of the peak area of m/z 483 to that for m/z 479 of the urine sample, and assumed that if the ratio of the peak area of m/z 483 to that for m/z 479 is B , we could calculate the recovery rate (%) as $B \times 100/A$.

Characteristics of subjects

The study group consisted of 18 bronchial asthmatic patients (seven females, 11 males), which excluded AIA patients, and 18 healthy subjects (11 females, seven males). The mean ages were 52.8 ± 15.6 years for the asthmatic patients and 48.1 ± 13.6 years for the healthy subjects. The asthmatic patients were classified into 13 atopic and five non-atopic. All the asthmatic patients were in a clinically stable condition. None of the asthmatic patients were receiving systemic corticosteroids or a 5-lipoxygenase inhibitor, and 17 out of the 18 asthmatic patients were using inhalation steroids (median, 800 μ g equivalent to BDP/day; range, 400–2400 μ g). Asthma severity was classified as follows: mild persistent asthma in three patients, moderate persistent asthma in nine patients, and severe persistent asthma in six patients.

Table 1. Clinical characteristics of patients

	Aspirin-induced asthma (n = 7)	Aspirin-tolerant asthma (n = 6)	P-value
Age (years)	49.9 ± 19.4	45.5 ± 18.0	NS
Male/female	3/4	4/2	NS
Atopy/non-atopy	2/5	3/3	NS
Duration of asthma (years)	11.7 ± 15.9	6.8 ± 4.0	NS
Severity	NS		
Mild persistent asthma	2	3	
Moderate persistent asthma	3	3	
Severe persistent asthma	2	0	
FEV ₁ (% predicted)	82.5 ± 14.3	99.2 ± 21.4	NS
Blood eosinophils (/mm ³)	956 ± 738	288 ± 240	NS
Serum IgE (IU/mL)*	312 (18–925)	199 (20–2470)	NS
Dose of inhalation steroids (µg/day)*	800 (0–1600)	400 (0–800)	NS
No. of patients receiving prednisolone	0	0	
No. of patients receiving leukotriene receptor antagonist	5	1	
No. of patients receiving salmeterol	0	0	

*Median (range).

NS, non-significant.

Permission to conduct the study was obtained from the National Sagami Hospital Ethics Committee and all subjects gave their informed consent. Urine samples were collected in polypropylene tubes containing 4-hydroxy-TEMPO and stored at -35°C until analysis. Urine was analysed within a year.

Aspirin challenge test

The study group consisted of seven patients with AIA and six patients with aspirin-tolerant asthma (ATA). All patients were in a clinically stable condition. All medications were stopped for at least 12 h prior to the challenge test. At the time of the study, forced expiratory volume in 1 s (FEV₁) exceeded 70% of the predicted value. The characteristics of the patients receiving aspirin challenge test are summarized in Table 1. There were no significant differences between the two groups in any of the parameters.

The challenge test was performed at about 9:00 to 12:00 hours as previously reported [2, 11]. Briefly, urine samples were collected at the beginning of the study. After intravenous injection of 1 mL of saline, if FEV₁ did not change by more than 10% compared with the pre-challenge baseline, double doses of lysine aspirin (12.5, 25, 50, 100, and 200 mg equivalent of aspirin) were intravenously administered. FEV₁ was recorded every 10 min after aspirin administration and the time interval between administrations of increasing doses was 30 min. The challenge test was stopped when FEV₁ decreased by 20% or more from the baseline. ATA patients did not exhibit changes in respiratory function even after receiving the highest dose of aspirin. Urine samples were collected during the following periods: 0–3, 3–6, 6–9, and 9–24 h after the onset of bronchoconstriction.

Quantification of urinary LTE₄

Urinary LTE₄ was quantified by enzyme immunoassay after purification by HPLC as reported previously [17].

Statistical analyses

Data were expressed as the mean ± SD unless otherwise specified. Differences between groups were analysed by the Mann–Whitney *U*-test. A non-parametric statistical test was performed using Friedman's test for time-course experiments. When the test indicated significant differences, the Tukey-type test was used to compare the data. Correlation between parameters was analysed using Spearman's rank correlation test. A *P*-value of less than 0.05 was regarded as statistically significant.

Results

Verification of the presence of urinary LTB₄ after hydrolysis with β-glucuronidase

For LTB₄ identification by GC-MS-SIM, a large volume of urine was hydrolysed by β-glucuronidase as described above and the eluate from HPLC was derivatized to the LTB₄ pentafluorobenzyl ester. After the LTB₄ pentafluorobenzyl derivative was purified by HPLC using a normal-phase column [19], the derivative was converted to its corresponding di-trimethylsilyl ether derivative. When the ions at *m/z* 389 and *m/z* 479 were monitored by GC-MS-SIM, the peak area ratio of the ion at *m/z* 389 to that at *m/z* 479 was 0.329 ± 0.003 ($n = 4$) for the authentic LTB₄ derivative. When the samples extracted from urine were analysed, the peak having two fragment ions appeared at the same retention time as that of the authentic LTB₄ derivative. The peak area ratio of the ion at *m/z* 389 to that at *m/z* 479 was 0.336 ± 0.008 ($n = 3$), which agreed with that of authentic LTB₄. This observation suggests that the presence of LTB₄ can be confirmed based on mass spectral patterns in addition to retention times on gas chromatography.

Exogenously administered LTB₄ is not excreted into the urine [20]. However, measurable concentrations of urinary LTB₄ were obtained in this study. To ascertain whether LTB₄ is present in human urine or whether the observed concentrations resulted from the cross-reactivity of the antibody for other compounds, we measured the peak area ratio of the LTB₄ derivative in urine by GC-MS-SIM without enzymatic hydrolysis. The peak area ratio was 0.344, confirming the presence of LTB₄ in human urine (Fig. 1).

Quantification of urinary LTB₄ by enzyme immunoassay in combination with HPLC

Rates of LTB₄ recovery from urine The average recovery rate was $72.6 \pm 3.2\%$ ($n = 6$) after all the purification methods. LTB₄ was stable during incubation without β-glucuronidase and the recovery rate was $99.0 \pm 1.3\%$ after incubating overnight at 37°C . However, LTB₄ concentration decreased to 80% of the control ($80.6 \pm 2.9\%$, $n = 4$) after incubation of LTB₄ in the presence of β-glucuronidase, thus

indicating that concentration of LTBG may be underestimated by approximately 20%.

Accuracy of the method The amount of LTB₄ was 13.7 pg when hydrolysing urine with β -glucuronidase. This amount shows endogenous LTB₄ and LTB₄ generated from LTBG by hydrolysis. Using three urine samples to which 42 pg of LTB₄ was added, and which were measured by the same method, we obtained the following results: 72.1, 52.2 and 61.3 pg. The rates of recovery from the urine sample with 55.7 pg (13.7 + 42 pg) of LTB₄ were 129%, 93.4%, and 110% ($110.8 \pm 17.8\%$). Similarly, the LTB₄ recovery was $116.1 \pm 12.5\%$ when 120 pg of LTB₄ was added to the pooled urine samples. The accuracy of this method was within acceptable limits.

The precision of the method was evaluated by analysing eight aliquots from the same urine specimen on the same occasion. Four aliquots were analysed without hydrolysis and the remaining were analysed with hydrolysis using β -glucuronidase. The endogenous LTB₄ concentration was 25.0 ± 2.9 pg/mL. After hydrolysis, the LTB₄ concentration was 41.7 ± 4.7 pg/mL. The coefficient of variation calculated as a measure of intra-assay variation was about 11% in the case of analysis with or without hydrolysis.

Urinary concentrations of LTB₄, LTBG and LTE₄ in asthmatic patients and healthy subjects

The bronchial asthmatic patients, which excluded AIA patients, showed significantly higher urinary LTBG concentrations than the healthy subjects (median, 3.32 pg/mg creatinine [range, 0.14–10.5] vs. 5.37 pg/mg creatinine [range,

1.2–13], $P = 0.0159$; Fig. 2), although there was substantial overlapping between the two groups. Significant difference in the concentration of urinary LTE₄, a confirmed marker for LTC₄ production *in vivo*, was also observed between the two groups (median, 35.7 pg/mg creatinine [range, 9.9–91.9] vs. 81.6 pg/mg creatinine [range, 19.2–255.3], $P = 0.014$; Fig. 2). There was no significant difference in urinary LTB₄ concentration between the two groups (median, 0.59 pg/mg creatinine [range, 0.3–4.3] vs. 0.99 pg/mg creatinine [range, 0.14–5.1], $P = 0.232$; Fig. 2). Moreover, no significant correlation between urinary LTE₄ and urinary LTBG concentrations was observed in the asthmatic patients when the data were analysed by Spearman's rank correlation test ($r = 0.040$, $P = 0.865$).

Baseline urinary LTBG and LTE₄ concentrations in AIA patients

As supported by previous reports, the AIA patients ($n = 7$) had a significantly higher urinary LTE₄ concentration than the ATA patients ($n = 6$) at the pre-challenge baseline (median, 331 pg/mg creatinine [range, 75–4946] vs. median, 54.5 pg/mg creatinine [range, 27.8–255.7], $P < 0.01$). In contrast, the two groups showed no significant difference in the concentrations of LTBG (median, 5.6 pg/mg creatinine [range, 3.6–18.9] for the AIA patients vs. median, 5.32 pg/mg creatinine [range, 0.46–8.7] for the ATA patients) and LTB₄ (median, 2 pg/mg creatinine [range, 0.6–6.4] for the AIA patients vs. median, 0.95 pg/mg creatinine [range, 0.42–2.1] for the ATA patients). There was no significant correlation between the basal concentrations of LTE₄ and LTBG in seven AIA patients ($r = 0.107$, $P = 0.819$).

Changes in urinary LTBG and LTE₄ concentrations after intravenous aspirin challenge in AIA and ATA patients

The seven AIA patients showed a greater than 20% decrease (21.1–30%, mean 26%) in FEV₁ after stimulation with a cumulative dose of 25–75 mg of aspirin. There was no significant correlation between a decrease in FEV₁ and the maximal concentrations of urinary eicosanoids. The number of patients may be too small to allow us to determine the correlation between a decrease in FEV₁ and urinary eicosanoid concentrations.

In all AIA patients, the intravenous injection of aspirin induced significant increases in both urinary LTBG and LTE₄ concentrations at the 0–3 and 3–6 h collection periods compared with the baseline concentrations (Fig. 3). The urinary

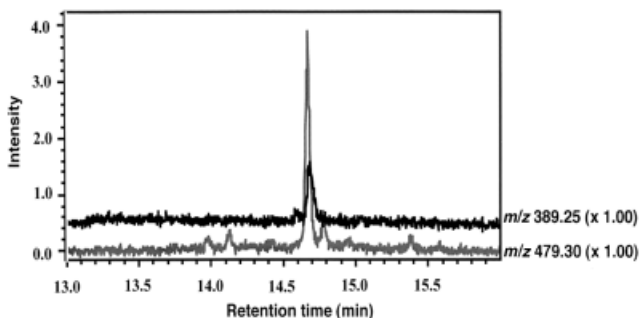


Fig. 1. Detection of leukotriene B₄ as pentafluorobenzyl ester di-trimethylsilyl ether derivative in human urine by gas chromatography-mass spectrometry-selected ion monitoring.

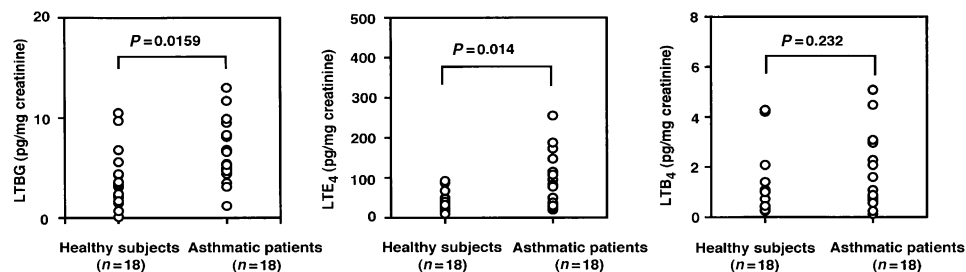


Fig. 2. Urinary concentrations of LTBG (left), LTE₄ (center) and LTB₄ (right) in asthmatic patients and healthy subjects. LTBG, LTB₄ glucuronide; LTE₄, leukotriene E₄; LTB₄, leukotriene B₄.

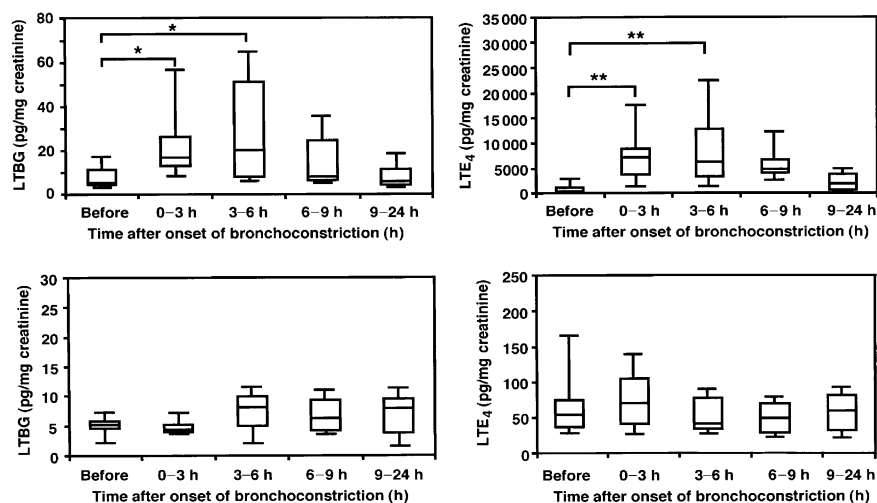


Fig. 3. Changes in urinary LTBG (left) and LTE₄ (right) concentrations in patients with aspirin-intolerant asthma (upper) and aspirin-tolerant asthma (lower) after intravenous aspirin challenge. Data are presented as box plots displaying medians and inter-quartile ranges. In the box plots, the lower boundary indicates the 25th percentile. The line within the box indicates the 50th percentile (median) and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles, respectively. The results were analysed using Friedman's test with a *post hoc* test. *Significantly different from the baseline concentration ($P < 0.05$). **Significantly different from the baseline concentration ($P < 0.01$). LTBG, LTB₄ glucuronide; LTE₄, leukotriene E₄; LTB₄, leukotriene B₄.

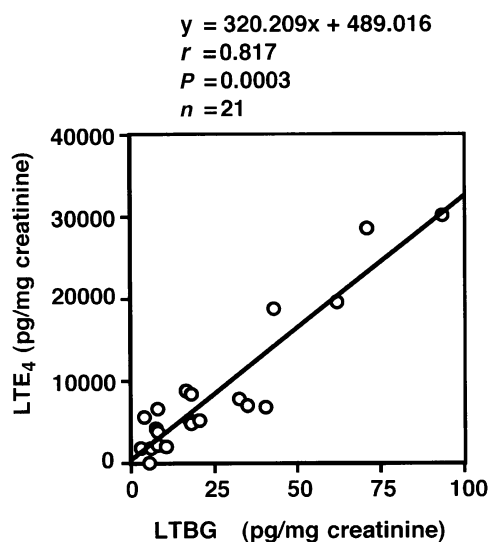


Fig. 4. Correlation between urinary LTBG and LTE₄ concentrations at the 0-3, 3-6 and 6-9 h collection periods after intravenous aspirin challenge in aspirin-intolerant asthma patients. LTBG, LTB₄ glucuronide; LTE₄, leukotriene E₄.

LTBG concentration increased from the baseline to 17.4 pg/mg creatinine (median [range, 5.7-93.7], $P < 0.05$) at the 0-3 h collection period and to 20.7 pg/mg creatinine (median [range, 3.2-71.2], $P < 0.05$) at the 3-6 h collection period. Similarly, the urinary LTE₄ concentration increased significantly from the baseline concentration at the 0-3 h collection period (median, 6932 pg/mg creatinine [range, 222-30176], $P < 0.01$) and at the 3-6 h collection period (median, 6696 pg/mg creatinine [range, 1859-28560], $P < 0.01$). Urinary LTB₄ concentration did not change significantly after aspirin challenge in the AIA patients. After aspirin challenge of six ATA patients, urinary LTBG and urinary LTE₄ concentrations did not change significantly in 24 h (Fig. 3).

Urinary LTE₄ concentration reached the peak in the first 3 h in four patients and during the collection period of 3-6 h in the remaining three patients. The periods in which the maximum LTE₄ and LTBG concentrations were reached

coincided in six of seven AIA patients. Both urinary LTBG and LTE₄ concentrations almost returned to their baseline concentrations at the 9-24 h collection period. When the data from seven AIA patients during the 0-3, 3-6 and 6-9 h collection periods were taken together, a significant linear correlation was found between urinary LTBG and LTE₄ concentrations (Fig. 4, $r = 0.817$, $P = 0.0003$, $n = 21$).

Discussion

The LTBG concentration was approximately 3.8 pg/mg creatinine (median) in urine samples from the healthy subjects, suggesting that the urinary LTBG concentration is approximately 10-fold lower than the concentration of urinary LTE₄, which is an established *in vivo* marker of cyst-LT production. Supposing that the metabolism of endogenously produced LTB₄ is the same as that of exogenously administered LTB₄ [15], namely, about 0.2% of endogenous LTB₄ is excreted into the urine as LTBG and the total amount of creatinine excreted is 1500 mg/day, the calculated whole-body LTB₄ production may be 2.85 µg/day. If metabolic clearance systems including liver function and UDP-glucuronosyl transferase activity were not altered in the disease state, urinary LTBG concentration provides a new and easily performed method to monitor whole-body LTB₄ production.

It has been inferred from chromatographic results that various glucuronide conjugates of LTB₄ exist in human urine [15]. We do not know whether each conjugate is sufficiently cleaved by hydrolysis although Karin et al. [15] reported that LTBG hydrolysis is completed in 20 h.

In spite of the findings that exogenous LTB₄ is not excreted into the urine [20], the analysis by GC-MS-SIM confirmed the presence of LTB₄ in human urine. The most likely source of urinary LTB₄ may be the kidney, where LTA₄ hydrolase is present [21].

Our data are the first to demonstrate that, similar to LTE₄ concentration, LTBG concentration increases in urine after aspirin challenge of AIA patients, suggesting that aspirin challenge results in the increased production of LTB₄ in AIA patients. On the other hand, urinary LTB₄ concentration did

not increase after aspirin challenge in AIA patients. Ferreri et al. [22] reported that following oral aspirin challenge of AIA patients, LTC₄ concentration in nasal lavage fluid increased but LTB₄ concentration in nasal lavage fluid remained unchanged. These results indicate that LTB₄ itself is not an appropriate marker of LTB₄ production, probably because it is rapidly degraded.

It was reported that aspirin administration to AIA patients increased urinary concentrations of not only LTE₄ but also 9 α , 11 β -PGF₂ and methylhistamine [2, 23]. Furthermore, an increased concentration of plasma 9 α , 11 β -PGF₂ following aspirin administration to AIA patients was also reported [10]. These findings support the possibility that LTC₄ may be produced by activated mast cells in AIA patients following aspirin administration, because PGD₂ is the predominantly produced PG by human mast cells and histamine is produced by mast cells and basophils. On the other hand, it is known that mast cells produce only small amounts of LTB₄ [24–26]. It is of interest to identify cells producing LTB₄ and to clarify the underlying production mechanism. It is known that eosinophils fail to produce LTB₄ [27, 28]. Taking also into consideration the absence of a significant increase in the concentration of 3-bromotyrosine produced by activated eosinophils, as demonstrated in our previous study [11], the possibility of LTC₄ production by activated eosinophils may as well be eliminated.

Some probable mechanisms underlying LTB₄ production are assumed. (1) Monocytes and macrophages or cells capable of producing both LTB₄ and LTC₄ are activated. Macrophages are also capable of producing PGD₂ [29, 30]. (2) In addition to mast cells, cells capable of LTB₄ production, such as monocytes, macrophages and neutrophils, are also activated simultaneously. However, our previous study showed that the urinary concentration of 3-chlorotyrosine known to be produced by activated neutrophils remains unchanged despite the increased urinary LTE₄ concentration following aspirin administration to AIA patients [11]. Based on these findings, there seems to be little possibility of neutrophil activation. (3) The possibility of LTB₄ and LTC₄ production by transcellular biosynthesis is also assumed [31–33]. That is, LTA₄ is released into the extracellular space along with LTB₄, and LTA₄ is converted to LTC₄ by cells that express LTC₄ synthase, such as mast cells, eosinophils, platelets [34–36] and endothelial cells [37–39]. Nasser et al. [40] reported that large numbers of eosinophils and mast cells are present in tissues obtained from AIA patients by bronchial biopsy compared with those in tissues obtained from ATA patients. Cowburn et al. [41] reported that counts of cells expressing LTC₄ synthase are significantly higher in bronchial biopsies from AIA patients than those from ATA patients. On the basis of these reports, the possibility of transcellular biosynthesis of LTB₄ and LTC₄ is also considered. On the other hand, it is also assumed that in relation to LTC₄ production by mast cells, LTA₄ released into the extracellular space may be transformed to LTB₄ by neighbouring cells such as epithelial cells expressing LTA₄ hydrolase [42].

In summary, further studies are necessary to elucidate the mechanism underlying LTC₄ and LTB₄ production following aspirin administration to AIA patients. The possibility that LTBG is produced by events starting from mast cell

activation cannot be excluded. It was reported that an allergen challenge of patients with bronchial asthma leads to an increased urinary LTE₄ concentration [43–47]. It was suggested that this increased urinary LTE₄ concentration following the allergen challenge is due to mast cell activation by the IgE antibody. The determination of changes in urinary LTE₄ and LTBG concentrations after the allergen challenge of patients with bronchial asthma may contribute to the clarification of whether mast cells are the main cellular source of urinary LTBG. On the other hand, the increase in urinary LTE₄ concentration following the inhalation allergen challenge of atopic patients was lower than the increase in LTE₄ concentration following the intravenous aspirin challenge of AIA patients. These findings also suggest another possibility that LTBG concentration shows only minute changes that are undetectable. Investigation of this possibility is in progress in our laboratory.

Since we used the intravenous route for aspirin challenge, it is impossible to identify the tissue that produced eicosanoids. In this study, we present the whole-body production of eicosanoids in AIA patients. When we used the inhalation route for aspirin challenge, eicosanoids were produced only in the lungs; therefore, it is necessary to confirm in future studies whether LTBG is also produced only in the lungs. However, an increase in urinary LTE₄ concentration in AIA patients challenged via the inhalation route was not as large as that via the intravenous route, and the increase in LTBG concentration is not expected to be significant; therefore, it may be difficult to obtain clear differences.

No correlation between pre-challenge LTE₄ and LTBG concentrations was observed. When statistical analysis for correlation was repeated for 20 AIA patients, which included 13 AIA patients added to the original seven AIA patients, no correlation between the two was observed. It seemed that LTC₄ overproduction observed in AIA patients in the stable condition did not involve LTB₄ overproduction. Although it may be proved unreasonable to compare baseline concentrations of metabolites whose metabolic pathways and metabolic rates differ from each other, it is difficult to assume the sustained production of a large amount of LTC₄ alone if the same types of cell produce LTC₄ and LTB₄. Only cells that produce LTC₄ may be subject to continuous activation in the clinically stable state. On the other hand, as shown in Fig. 3, there was a significant correlation between increased urinary concentrations of LTC₄ and LTB₄ following aspirin administration. LTBG and LTE₄ concentrations peaked simultaneously, namely, LTGB and LTE₄ concentration changes followed the same time course. These results suggest that both LTs produced following aspirin administration are closely associated with each other.

In conclusion, the estimation of urinary LTBG concentration may be a very useful method of assessing the biosynthesis of LTB₄, whose role in bronchial asthma remains to be elucidated. The determination of LTBG concentration indicated that not only LTC₄ production but also LTB₄ production increased in AIA patients following aspirin challenge. These findings provide significant information regarding cells involved in the pathogenesis of AIA. The elucidation of a more important issue, that is, the mechanism underlying the production of both LTs, is required in further studies.

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