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Exercise-Induced Urinary Excretion of Leukotriene E_4 in Children with Atopic Asthma

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ABSTRACT. Urinary levels of leukotriene (LT) E_4, a stable end-product of LTC_4 and LTD_4, were measured before and after exercise in 10 children with severe asthma and seven children with moderate asthma using HPLC and RIA to clarify the relationship of LT to the severity of asthma and to the degree of bronchospasm in exercise-induced asthma. The urinary LTE_4 level significantly increased after exercise in the severe asthma group, but not in the moderate asthma group (14.3 ± 14.5 to 24.3 ± 20.6 *versus* 19.6 ± 12.3 to 17.6 ± 10.8 ng/mmol creatinine, $p < 0.05$). The urinary LTE_4 level increased in 10 patients (eight with severe asthma), and it decreased in seven patients (five with moderate asthma). A significant difference in the degree of bronchospasm after exercise (as shown by the maximal % fall in the peak expiratory flow rate), was seen when patients with increased urinary LTE_4 excretion were compared with those with decreased excretion (60.4 ± 17.3 *versus* 24.1 ± 14.3%, $p < 0.01$). Our findings suggest that exercise-induced asthma, or at least a subtype of exercise-induced asthma, may partly develop through the release of LTC_4. (*Pediatr Res* 29: 455–459, 1991)

Abbreviations

LT, leukotriene
EIA, exercise-induced asthma
PEFR, peak expiratory flow rate

The development of bronchoconstriction several minutes after vigorous exercise is a hallmark of EIA (1). Although it is widely accepted that either heat or water loss from the airways during exercise is the stimulus for EIA, the mechanism by which these stimuli induce constriction of the bronchial smooth muscle remains unknown (1). One hypothesis is that EIA develops after the release of chemical mediators, such as histamine and neutrophil chemotactic factor, from cells located in the bronchial mucosa (2, 3). However, the involvement of histamine release in EIA is controversial because several laboratories have failed to demonstrate histaminemia in this condition (4), suggesting that plasma histamine changes may be related to the associated basophilia and sample handling rather than to intrapulmonary mast cell activation (5).

The sulfidopeptide LT (C_4, D_4, and E_4) are another major group of mast cell-derived bronchoactive mediators that are several hundred times more potent than histamine. They have been suggested to have an important role not only in asthma but also in other diseases, such as neonatal persistent pulmonary hypertension and acute viral respiratory infections in children (6–8). However, little is known about the role of the sulfidopeptide LT in EIA. Arterial blood sampling is considered necessary to preclude the artificial formation of these sulfidopeptide LT (9), inasmuch as the high concentrations reportedly detected in plasma derived from venous blood are probably due to cellular injury during sample collection (9, 10). This seems to be an especially big problem in blood collection from infants and young children. Furthermore, the initial product of sulfidopeptide LT, LTC_4, is reportedly unstable even when stored at low temperatures (11), whereas LTE_4 is relatively more stable than LTC_4 or LTD_4 in urine, even at 37°C (12). Therefore, to examine the role of these sulfidopeptide LT in EIA, we measured urinary LTE_4 concentrations before and after exercise in children with asthma of varying severities and explored the relationship of the urinary LTE_4 concentration to the severity of asthma in EIA.

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MATERIALS AND METHODS

Thirty-six children with a history of EIA, positive skin test to common allergens, and elevated IgE levels were entered into this study after informed consent was obtained. A control study with healthy children was not performed, because we could obtain informed consent from only a small number of healthy children and their parents. The children were classified as having either mild, moderate, or severe asthma, according to the criteria of the Japanese Research Group for Pediatric Allergy (Table 1). The classification of the patients was performed independently by other doctors unrelated to the measurement of urinary LTE_4, before the urinary LTE_4 assay. The children with mild asthma could not produce enough urine for the assay. Urinary LTE_4 levels were measured in 10 children with severe asthma (age range: 7–12 y, mean: 9 y) and in seven children with moderate asthma (age range: 7–10 y, mean: 9 y), all of whom produced the required amount of urine (more than 60 mL) both before and after exercise. None of our subjects had used steroids or disodium chromoglycate for at least several months before the start of this investigation, with the exception of three children in the moderate asthma group who were using disodium chromoglycate. Subjects were asked to abstain from the intake of any medications for 12 h before the study.

Exercise testing. Exercise was performed on a treadmill for 6 min with the speed set at 6.0 km/h and the incline set at a 10°. PEFR was measured using a peak flow meter (AS-500, Minato Medical Science, Osaka, Japan) before exercise and after exercise for up to 30 min. The best of three consecutive PEFR readings at each time point was used for subsequent analysis. All procedures were undertaken at ambient room temperature.

Collection of urine samples. Subjects were asked to pass urine 2 h after exercise. Urine was actually collected from 1 to 2 h after exercise, and was stored at −60°C until later use.

Measurement of LTE_4. Urinary LTE_4 was measured essentially as described by Verhagen *et al.* (13), except for an additional purification step using a Sep-Pak NH_2 cartridge (Millipore Waters Associates, Milliford, MA). Urine samples were tested in duplicate. Samples were allowed to thaw immediately before the assay, 6000 dpm of ^{3}H-labeled LTE_4 (56 Ci/mmol, Amersham International, Buckinghamshire, UK) was added as an internal standard, and centrifugation was performed at 45 000 × *g* for 10 min at 4°C. The supernatant was first applied to a Sep-Pak C_{18} cartridge (Millipore Waters Associates), and then washed with 10 mL of water and followed by 5 mL of methanol/water (50:50, vol/vol), after which LT were eluted with 3 mL of methanol. The 3-mL methanol fraction was applied again to a Sep-Pak NH_2 cartridge. After washing the cartridge with 10 mL of methanol, the LT were finally eluted into 15 mL of a 0.5% acetic acid methanol solution (vol/vol). Then the sample was dried under a stream of nitrogen gas and its volume was adjusted to 200 μL with 65% methanol. The sample was then injected into a C_{18} reverse-phase HPLC column (Nacalai Tesque, Kyoto, Japan) and eluted through an HPLC system (Millipore Waters Associates) at a constant flow rate of 1 mL/min, using a mixture of methanol/water/acetic acid (65:35:0.1) adjusted to pH 4.9 with ammonia. The fractions having the same elution time as the synthetic LTE_4 were collected, and the immunoreactive LTE_4 content was determined by RIA.

RIA for LTE_4. The RIA was carried out as described in the manufacturer's instructions. The LT $C_4/D_4/E_4$ [^{3}H] assay kit was purchased from Amersham International. A standard solution of LTE_4 was dissolved in the same HPLC solvent with the samples to compensate for loss by evaporation during processing for the RIA. Both the standard solution and the fractions obtained from HPLC were dried in a vacuum, and the precipitates were resuspended in the immunoassay buffer. LTE_4 was used as the radioligand and the samples were quantified against an LTE_4 standard curve. All values are given as the mean ± SD.

The recovery of the radioactive LTE_4 added to urine samples was 32 ± 7% after extraction and HPLC. The precision of the overall procedure was checked by repeating the analysis on the same urine samples pooled from different patients with and without the addition of a known amount of synthetic LTE_4 immediately before extraction. An added LTE_4 concentration of 200 pg/mL was detected as an increase of 173 ± 28 pg/mL, with recovery of 38 ± 6% of the radioactivity. The intraassay and the interassay variations were 7 and 9%, respectively, using pooled urine samples from healthy adults. Based on the intraassay variation of 7%, the urinary LTE_4 level was considered to be increased when the value after exercise was 1.07 times greater than that before exercise, and to be decreased when the value before exercise was 1.07 times greater than that after exercise.

Statistical analysis. The unpaired *t* test was used for the statistical comparison of urinary LTE_4 levels and the maximal % fall in PEFR between the moderate and severe asthma groups, and also for comparison of the recovery of radioactive LTE_4 from the urines and the maximal % fall in PEFR between children with decreased and increased urinary LTE_4 excretion both before and after exercise. The paired *t* test was used for the statistical comparison of urinary LTE_4 excretion between before and after exercise groups, in both the moderate and severe asthma groups, and also for comparison of the recovery of radioactive LTE_4 before and after exercise in children with decreased and with increased urinary LTE_4 excretion. A p value < 0.05 was considered significant.

Table 1. *Criteria of Japanese Research Group for Pediatric Allergy*

	Criteria
Grading of asthma attack:	
Mild	Minimal degree of wheezing or pulmonary obstruction
	No sleep interruption due to asthma
	Good exercise tolerance
	Good school attendance
Moderate	Moderate degree of wheezing or pulmonary obstruction, without orthopnea or cyanosis
	Sleep may be interrupted due to asthma
	Exercise tolerance diminished
	School attendance may be affected
Severe	Substantial degree of wheezing or pulmonary obstruction with orthopnea, sometimes associated with cyanosis
	Marked sleep interruption
	Poor exercise tolerance
	Poor school attendance
Classification of severity of asthma based on grading of attack:	
Mild	Less than 10 mild attacks per year or up to five moderate attacks per year
Moderate	More than 11 mild attacks per year or from six to 10 moderate attacks per year or up to five severe attacks per year
Severe	More than six severe attacks per year or more than 11 moderate attacks per year, with steroid dependence or with an episode of disturbance of consciousness due to asthma, regardless of the number or type of attacks

RESULTS

Chromatography. Figure 1 shows a representative immunochromatogram of a urinary extract together with the radioactive chromatogram from ^{3}H-LTE_4 and the UV absorbance at 280

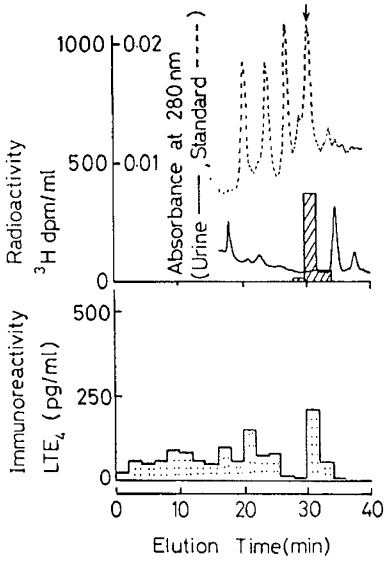

Fig. 1. Representative reverse-phase HPLC chromatogram of a urine sample. Urine was extracted with Sep-Pak C_{18} and Sep-Pak NH_2 cartridges and resolved by HPLC. LTE_4 immunoreactivity as determined by RIA (*dotted columns*) is shown in the *lower panel*. Radioactivity from a ^{3}H-labeled internal standard (*hatched columns*) is shown with UV absorbance at 280 nm (*solid line*) together with that of synthetic LTB_4, LTC_4, LTD_4, and LTE_4 (*broken line*) in the *upper panel*. An *arrow* indicates the peak corresponding to synthetic LTE_4.

nm. For reference, a typical reverse-phase HPLC separation of a mixture of synthetic LT (LTB_4, LTC_4, LTD_4, and LTE_4) detected at 280 nm is also shown in *broken lines*. The largest radiochromatogram peak approximately coincided with the peak for synthetic LTE_4, with a retention time of about 31 min, and significant immunoreactivity was found in the corresponding fraction.

Differences between moderate and severe asthma. The data for the moderate and severe asthma groups on urinary LTE_4, excretion, radioactive LTE_4 recovery, peak flow as % of the age-predicted PEFR, and maximal % fall in PEFR are shown in Tables 2 and 3. The changes in the urinary LTE_4 excretion and maximal % fall in PEFR are summarized in Table 4. There was no significant difference between the moderate and severe asthma groups with regard to the urinary LTE_4 levels before and after exercise, although the maximal % fall in PEFR of the severe group was significantly greater than that of the moderate group. However, a significant increase in urinary LTE_4 excretion after exercise was noted in the severe asthma group ($p < 0.05$; Fig. 2*B*).

Relationship between changes in urinary LTE_4 level with exercise and maximal % fall in PEFR. When both groups were considered ($n = 17$), there was no significant correlation between urinary LTE_4 levels with exercise and the maximal % fall in PEFR, regardless of whether the change in the urinary LTE_4 levels was calculated as a ratio or in absolute values.

Among the 17 subjects, urinary LTE_4 levels increased in 10 (eight with severe asthma and two with moderate asthma). Conversely, the urinary LTE_4 levels decreased in seven subjects (five with moderate asthma and two with severe asthma). Thus, the classification of the subjects on the basis of changes in the urinary LTE_4 correlated well with that based on asthma severity. There was a significant difference in the maximal % fall in PEFR between the groups with increased and decreased urinary LTE_4 excretion (60.8 ± 21.8 *versus* $24.1 \pm 14.3\%$, $p < 0.01$) (Fig. 3).

Urinary radioactive LTE_4 recovery from children with increased or decreased LTE_4 excretion. As shown in Table 5, there was no significant difference between the urinary radioactive LTE_4 recovery in the children with decreased urinary LTE_4 excretion and that in those with increased urinary LTE_4 excretion before or after exercise. There was also no significant difference in either group when the recovery before and after exercise was compared.

DISCUSSION

The urinary recovery of radioactive LTE_4 in our experiment (about 30%) was relatively low compared with that reported for automatic extraction (40–60%) or that for manual extraction (about 50%) (14, 15). In a preliminary experiment, when urine was extracted only with a Sep-Pak C_{18} cartridge, the radioactive recovery was about 50%—comparable to that in other reports. However, the additional extraction step with a Sep-Pak NH_2 cartridge led to a substantial improvement in the immunochromatogram and UV-chromatogram of the urinary extracts, as well as to significantly lower urinary LTE_4 concentrations than those obtained by extraction only with a Sep-Pak C_{18} cartridge (16). Furthermore, the recovery of radioactive LTE_4 added to urine samples was relatively constant and reproducible, and there was no significant difference in the radioactive recovery between the urine from patients with increased and those with decreased urinary LTE_4 excretion. Therefore, we consider that our relatively low recovery rate was a result of intensive purification to ensure the specificity of the urinary LTE_4 assay, and was not due to an error in the assay method.

Our results showed that there was no significant correlation between the changes in urinary LTE_4 levels and the maximal % fall in PEFR in EIA. This lack of a correlation between these two factors was not necessarily unexpected and was compatible with the observations of Taylor *et al.* (14). They reported the urinary LTE_4 levels of asthma patients both after specific antigen challenge and during acute attacks, and found no correlation between urinary LTE_4 levels and airway obstruction as determined by PEFR. Our results and theirs are consistent with the concept that both mast cell mediator release and nonspecific airway reactivity are independently responsible for acute airway obstruction after antigen challenge or exercise loading in EIA (17). The change in the urinary LTE_4 levels, therefore, may not necessarily be correlated with airflow obstruction.

We found a nearly 2-fold significant increase in urinary LTE_4 levels in the patients whose clinical symptoms were classified as severe. Because LT can induce severe bronchoconstriction and vasoconstriction, as well as an increase in bronchial mucus production (6), it would seem reasonable to assume that EIA is partly due to mast cell activation with subsequent release of LT, histamine, and neutrophil chemotactic factor (2). However, it is also possible that the increased amounts of urinary LTE_4 in the

Table 2. *Prechallenge and postchallenge urinary LTE_4 levels, radioactive LTE_4 recovery, and peak flow in patients with moderate asthma**

Patient no.	LTE_4 (ng/mmol creatinine)		Recovery of LTE_4 (%)		Predicted PEFR (%)		
	Pre	Post	Pre	Post	Pre	Post (lowest)	Maximal fall in PEFR (%)
1	30.7 (32.0, 29.4)	28.0 (26.7, 29.3)	26	34	102	100	2
2	2.1 (1.7, 2.5)	13.7 (11.6, 15.8)	35	47	93	81	13
3	15.0 (13.8, 16.2)	13.3 (12.4, 14.2)	28	23	106	85	20
4	32.0 (29.3, 34.7)	14.5 (11.3, 17.7)	33	28	95	80	16
5	36.0 (31.5, 40.5)	38.6 (34.5, 42.7)	23	27	121	33	73
6	14.7 (12.8, 16.6)	11.2 (10.4, 12.0)	43	36	113	89	24
7	6.8 (5.1, 8.5)	3.7 (2.9, 4.5)	29	41	118	87	26
Mean	19.6[a]	17.6[b]	31.0	33.7	107.0	79.3	24.9[c]
SD	12.3[a]	10.8[b]	6.2	7.8	10.1	19.9	20.9[c]

* Each pair of values in parentheses was obtained by duplicate RIA and each number above the parentheses represents the mean of these two values. Values with superscripts are shown in Table 4 for comparison.

Table 3. *Prechallenge and postchallenge urinary LTE_4 levels, radioactive LTE_4 recovery, and peak flow in patients with severe asthma**

Patient no.	LTE_4 (ng/mmol creatinine) Pre	LTE_4 (ng/mmol creatinine) Post	Recovery of LTE_4 (%) Pre	Recovery of LTE_4 (%) Post	Predicted PEFR (%) Pre	Predicted PEFR (%) Post (lowest)	Maximal fall in PEFR (%)
1	21.2 (17.6, 24.8)	45.7 (38.9, 52.5)	37	28	95	46	52
2	8.6 (7.4, 9.8)	11.0 (9.3, 12.7)	27	35	105	15	85
3	4.9 (3.9, 5.9)	1.3 (1.1, 1.5)	36	24	75	53	30
4	4.3 (3.6, 5.0)	39.3 (42.6, 36.0)	35	26	98	63	36
5	1.3 (1.0, 1.6)	1.7 (1.4, 2.0)	34	22	132	61	54
6	45.6 (41.0, 50.2)	64.6 (57.9, 71.3)	33	49	97	20	79
7	0.5 (0.4, 0.6)	10.6 (0.9, 12.3)	22	38	108	22	80
8	23.8 (21.0, 26.6)	39.3 (35.0, 43.6)	35	26	105	37	68
9	30.0 (27.0, 33.0)	23.4 (19.3, 27.5)	23	35	83	42	50
10	3.2 (2.8, 3.6)	6.2 (5.3, 7.1)	43	39	116	36	69
Mean	14.3[a]	24.3[b]	29.7	32.2	101.0	39.5	61.0[b]
SD	14.5[a]	20.6[b]	10.7	2.0	15.3	16.0	17.0[b]

* Values in parentheses were obtained by duplicate RIA and values above the parentheses represent the mean of the two values. Values with superscripts are shown in Table 4 for comparison.

Table 4. *Prechallenge and postchallenge urinary LTE_4 levels (ng/mmol creatinine) and maximal % fall in PEFR before and after exercise*

	Moderate asthma	Severe asthma	p*
Prechallenge	19.6 ± 12.3[a] (n = 7)	14.3 ± 14.5[a] (n = 10)	NS
Postchallenge	17.6 ± 10.8[b] (n = 7)	24.3 ± 20.6[b] (n = 10)	NS
Maximal % fall in PEFR	24.9 ± 20.9[c] (n = 7)	61.0 ± 17.0[c] (n = 10)	<0.01

* Comparison was made between the values for children with moderate asthma and those with severe asthma. Values with superscripts are shown in Table 2 or 3.

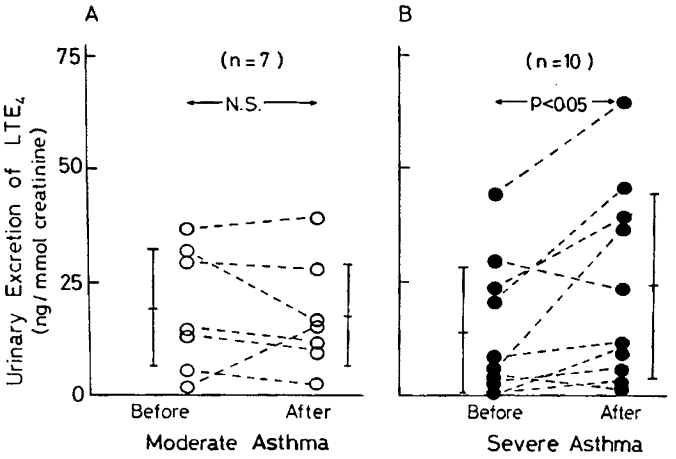

Fig. 2. Effect of exercise loading on urinary LTE_4 excretion in children with moderate asthma (*A*) and severe asthma (*B*). Significantly higher urinary LTE_4 concentrations are seen after exercise in the children with severe asthma, but not in those with moderate asthma.

severe asthma group could have been derived from another cellular source, inasmuch as the mast cell is known to not be the only source of LT (18). For example, endothelial cells and platelets in the presence of neutrophils are able to produce large quantities of LT *in vivo* under certain circumstances (19–21). Thus, we cannot completely rule out the possibility that the increased urinary LTE_4 excretion was secondary to marked bronchoconstriction (a mean 61.0% maximal fall in PEFR), because we only found a significant excretion of LTE_4 in the subjects with severe asthma.

The classification of the asthma patients was based on the clinical symptoms (Table 1) and not defined on the basis of changes in peak flow. Therefore, increased urinary LTE_4 excretion might be related to a subgroup of asthma patients with a greater decrease in peak flow after exercise rather than to severe asthma itself. This speculation is supported by the finding of a significantly greater decrease in peak flow in the patients with increased urinary LTE_4 excretion than in those with decreased urinary LTE_4 excretion. However, there were also two patients with greater than 30% of maximal fall in PEFR in the group with decreased urinary LTE_4 excretion. This discrepancy may perhaps be explained by accepting that both mediators released from mast cells and nonspecific airway reactivity are independently responsible for acute airway obstruction in asthma after antigen challenge or exercise loading (17). Other possibilities include the following: LT may play a role only in more severe bronchospasm; the urines may have been collected at the wrong time before the peak of LT excretion; the release of LT may not be important in bronchospasm after exercise; and our assay method may not have been able to detect changes in urinary excretion leading to milder bronchospasm.

There are only two reports, including that of Taylor *et al.*, on urinary LTE_4 excretion in humans (14, 15). The urinary LTE_4 levels before exercise in our study were not so different from those reported by Taylor *et al.* (mean ± SD: 23.8 ± 5.4 ng/mmol creatinine in 24-h urine samples collected from 29 healthy adults), except that our data showed a wider variation compared

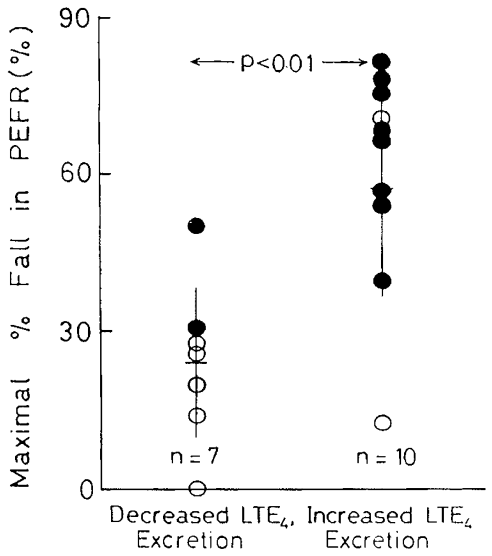

Fig. 3. Comparison of the maximal % fall in PEFR before and after exercise between children with decreased urinary LTE_4 concentrations and those with increased urinary LTE_4 concentrations. *Open and closed circles* indicate the values for moderate asthma and severe asthma, respectively. A significantly higher maximal % fall in PEFR was found in children with increased urinary LTE_4 excretion.

Table 5. *Recovery of radioactive LTE_4 from urine of patients with increased or decreased LTE_4 excretion during and after exercise*

	Decreased excretion	Increased excretion	p*
Prechallenge	31.0 ± 6.2 (n = 7)	32.5 ± 6.0 (n = 10)	NS
Postchallenge	31.8 ± 5.9 (n = 7)	33.5 ± 9.1 (n = 10)	NS
p†	NS	NS	

* Comparison was made between the values for children with decreased urinary LTE_4 excretion and those for children with increased urinary LTE_4 excretion.

† Comparison was made between the prechallenge and postchallenge values for the children with decreased urinary LTE_4 excretion and for the children with increased urinary LTE_4 excretion, respectively.

with values for normal adults (14). This wider variation may be ascribed to the relatively small sample number, effects of diurnal variation in LTE_4 excretion, or difference between adults and children. The mean urinary LTE_4 level after exercise in our severe asthma group (24.3 ng/mmol creatinine) was lower than the mean urinary LTE_4 excretion in 24 h in adult asthma patients (78.3 ng/mmol creatinine) (14). We probably need to take into consideration the frequency and timing of urine collection to explain the wider variation and lower mean LTE_4 level in our patients, because the excretion of urinary LTE_4 might be at a maximum more than 2 h after exercise. In an experiment involving i.v. injection of radioactive LTC_4 into healthy adults, 33% of the recovered radioactivity appeared in the urine. Furthermore, 44% of the total urinary radioactivity was excreted during the first hour after injection and was mainly found in the LTE_4 fraction (22). Also, in another experiment involving the inhalation of nonradioactive LTD_4 by healthy adults, approximately 50% of the total urinary LTE_4 was excreted during the first 2 h (13). Therefore, we tried to collect urine before exercise and 2 h after exercise. However, the dynamics of the urinary excretion of LTE_4 might be different in children or during EIA than those in healthy adults. Actually, a recent study showed that the urinary LTE_4 concentrations in asthma attacks induced by aspirin were maximal after 3 to 6 h and an average of 4.5 times higher than the concentrations before the asthma attacks, whereas they were less than twice the basal LTE_4 concentrations at 0 to 3 h, agreeing with our results (23).

In conclusion, we found that increased whole body production of LT occurs in a subgroup of EIA patients with more severe bronchospasm, supporting the idea that mast cell activation is involved in the mechanism of EIA. However, our results are not conclusive because of a lack of healthy controls and insufficiently frequent urine collection. Further studies are therefore needed to confirm our findings and to evaluate the effects of antagonists or inhibitors specific to LT on the prophylaxis and treatment of EIA.

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