

The challenge procedure influences the extent of allergen-induced urinary excretion of leukotriene E₄

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Summary

Background Cysteinyl-leukotrienes are central mediators in asthma and urinary leukotriene E₄ (LTE₄) is a reliable marker of their endogenous formation.

Objective This study tested the hypothesis that the procedure used for allergen bronchoprovocation influences the bronchoconstrictor response and the amount of LTE₄ excreted following allergen challenge.

Methods Seven atopic asthmatic men underwent two allergen bronchoprovocations 4 weeks apart. The same total dose of allergen was given at both sessions, cumulatively on one occasion and as a single dose at the other session. Urine was collected in hourly samples before and after challenge and LTE₄ was measured with previously validated methodology.

Results The mean ($\pm$ SE) drop in FEV₁ was not significantly different between the cumulative ($29 \pm 2.4\%$) and the single dose challenge ($25 \pm 2.8\%$). There was a significant increase in post-challenge levels of urinary LTE₄ after both sessions. The peak excretion of LTE₄ occurred 1 h following the maximal drop in FEV₁ for both challenges. However, the post-challenge increase in urinary LTE₄ was significantly larger at the cumulative session. In fact, the net increase (post-challenge minus prechallenge) of urinary LTE₄ was more than twofold higher after the cumulative session (AUC 0–3 h post-challenge: 46.7 ± 8.2 vs 22.1 ± 9.8 , $P < 0.05$).

Conclusion The peak excretion of urinary LTE₄ occurred within 2 h after the termination of either challenge but the magnitude of urinary excretion of LTE₄ was larger when cumulative challenge was performed. The findings are important to consider when designing studies where allergen-induced urinary excretion of LTE₄ is an outcome variable.

Keywords: allergen, bronchial provocation, enzyme immunoassay, urinary leukotriene E₄

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Introduction

The cysteinyl-leukotrienes (LTC₄, LTD₄ and LTE₄) have been established as important mediators in asthma [1]. There is accordingly increased urinary excretion of their pulmonary end-metabolite LTE₄ following challenge with allergen [2–8] and other indirectly acting bronchoconstrictive agents [7,9–11], as well as in association with spontaneous asthma attacks [2,8].

The magnitude of urinary excretion of LTE₄ after allergen challenge has, however, varied between investigations. Studies employing cumulative allergen challenges seem to have produced more consistent increases in urinary LTE₄ [7,12,13] than those where the subjects have been challenged by inhalation of one single dose of allergen [4,14–16]. In fact, some of the single dose studies did not find a significant post-challenge elevation in urinary LTE₄. As this tendency has also been observed between studies from the same laboratory where the method for measuring urinary LTE₄ has been identical ([12] vs [15] and [7] vs [16]), it seemed less likely that variability in analytical methodology explained the trend emerging from the literature. We therefore arrived at

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the hypothesis that the procedure used for the bronchial challenge may influence the urinary excretion of LTE_4 . Since the number of publications in this area altogether are few, and as it may be of practical consequences for studies where allergen-induced urinary LTE_4 is an endpoint, we decided to test our hypothesis. Specifically for allergen-challenge, we wanted to establish whether or not there was a difference between single dose and cumulative challenge with respect to the airway response and the urinary excretion of LTE_4 .

The study involved bronchial challenge of a group of atopic asthmatics with the same dose of allergen on two different occasions. At one session, allergen was inhaled in cumulatively increasing doses every 15 min until forced expiratory volume in 1 s (FEV_1) had decreased by 20%. At the other session, the same total allergen dose was given as one single inhalation. It was considered particularly important to use the same total dose of allergen during both sessions, because we have previously suggested that urinary excretion of LTE_4 is dose-dependent [7,11].

We have recently documented sustained elevation of urinary excretion of LTE_4 in atopic asthmatics displaying a late allergen-induced reaction [17]. A group of asthmatics with isolated early reactions was therefore selected, since it was felt that renewed synthesis of cysteinyl-leukotrienes and a second wave of bronchoconstriction in subjects with late reactions would obscure the questions we addressed in this particular study.

Materials and methods

Subjects

Seven non-smoking atopic asthmatic men with a mean age of 28 years (range 19–46) and a mean FEV_1 87% (range 76–106) of predicted participated in the study. The subject characteristics are shown in Table 1. They had all been established to display isolated early reactions in at least two allergen bronchoprovocations prior to this study. Their asthma was stable and controlled by inhaled β_2 -agonists as required. The study was approved by the Ethics Committee of the Karolinska Hospital and the participants gave informed consent.

Bronchial provocations and collection of urine samples

The protocols for allergen provocations which were followed in this study are published elsewhere [16,18]. Briefly, the patients were first challenged cumulatively with geometrically increasing doses of allergen every 15 min, until FEV_1 had decreased by 20% or more. The mean ($\pm$ SE) duration of the cumulative bronchoprovocation was 66 ± 7 min, and the mean number of doses of inhaled allergen were 4.1 ± 0.3 . At the following session, the patients inhaled the same total

Table 1. Subject characteristics

Subject number	Age (years)	FEV_1 (L)	Allergen	Dose (SQ)
1	46	76	Dog	71
2	24	93	Grass	710
3	19	106	Cat	210
4	33	88	Cat	710
5	28	79	Cat	213
6	23	80	Dog	71
7	25	87	Cat	710
Mean	28	87		259*
Range	19–46	76–106		71–710

*Geometric mean

dose of allergen (Table 1) using a single-dose challenge procedure. The challenges were separated by at least 4 weeks and allergen avoidance measures were instilled during the study. The same dosimeter (Spira Electro 2, Respiratory Care Center, Hämeenlinna, Finland) was used at both sessions.

Both challenges started at around 09.00 h with recordings of baseline pulmonary function. Urine was collected hourly with two portions before the start of the bronchoprovocation, and five or more portions after the challenge. Urine produced and sampled during the actual inhalation of cumulative allergen doses was not used in the analysis. The total volume of the urine samples was measured and 50-mL aliquots were immediately frozen. The samples were stored without preservatives at -20°C until analysis.

Analyses of urine samples

Urinary LTE_4 was measured using a sensitive enzyme immunoassay utilizing a rabbit polyclonal antiserum and acetylcholine esterase-linked LTE_4 tracer, as previously described [19]. Briefly, microtitre plates (96-wells) were coated with mouse monoclonal antirabbit IgG for 18 h at room temperature followed by addition of saturation buffer. After an additional 18 h at $+4^\circ\text{C}$ the saturation buffer was removed and the plates were ready to use. Solutions of LTE_4 standard, LTE_4 tracer and LTE_4 antiserum were prepared in EIA buffer and added in 50- μL aliquots to the wells. Unknowns were added in several dilutions. The plates were incubated overnight in darkness at room temperature, washed and supplied with the enzyme substrate (Ellman's reagent). Absorbance was measured at 414 nm. The standard curve ranged from 7.8 to 1000 pg/mL and the working detection limit of the assay was about 8 pg/mL. Accuracy, in terms of precision and specificity, of this assay has been validated in great detail and published elsewhere [19].

Creatinine was measured in all urine samples by a colourimetric alkaline picrate method using a standard procedure and commercially available kits. Results are expressed as ng LTE₄ per mmol of creatinine.

Materials

Rabbit polyclonal antiserum against LTE₄, acetylcholinesterase-linked LTE₄ tracer, synthetic LTE₄ standard, mouse monoclonal antirabbit IgG and Ellman's reagent were all purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Kits for analysis of creatinine were obtained from Sigma (St Louis, MO, USA). EIA buffer (100 mg sodium azide, 23.4 g sodium chloride, 370 mg tetra sodium EDTA and 1 g BSA per litre of 0.1 mol/L potassium phosphate buffer pH 7.4) was prepared in the laboratory. Allergen extracts and diluent for bronchial provocations (Aquagen) were from ALK Laboratories, Copenhagen, Denmark.

Statistics

Results are reported as mean $\pm$ SE. For calculation of the net increase in urinary LTE₄ excretion, the concentration in the portion collected immediately before the start of the bronchoprovocation was used as baseline and subtracted from the respective post-challenge values. From the individual data points for post-challenge net increase in urinary LTE₄, the area under the curve (AUC) was calculated for both sessions using the trapezoidal rule for integration. The data for pulmonary function and urinary LTE₄ were found to be normally distributed, and comparisons between groups were performed with Student's paired *t*-test. Differences were considered significant if $P < 0.05$. The statistical calculations and AUCs were calculated by the use of a validated statistical software package for personal computers (Sigma-SuiteTM, Jandel Scientific, Sansolito, CA, USA).

Results

The mean drop in FEV₁ was not significantly different between the two sessions, 25 ± 2.8 and $29 \pm 2.4\%$ after the single dose and cumulative challenge, respectively.

The prechallenge levels of urinary LTE₄ were not different before the two sessions (Fig. 1). There was a significant increase in urinary LTE₄ above baseline levels following both provocations (Fig. 1). The peak increase in urinary LTE₄ consistently occurred in the first or second sample collected after the peak drop in FEV₁ (Fig. 1), with no difference in mean time for peak increase in urinary LTE₄ between the two sessions (77 ± 10 min and 60 ± 12 min at the cumulative and single dose challenges, respectively).

The post-challenge increase in urinary LTE₄ was, however, larger after the cumulative session (Fig. 1). There was

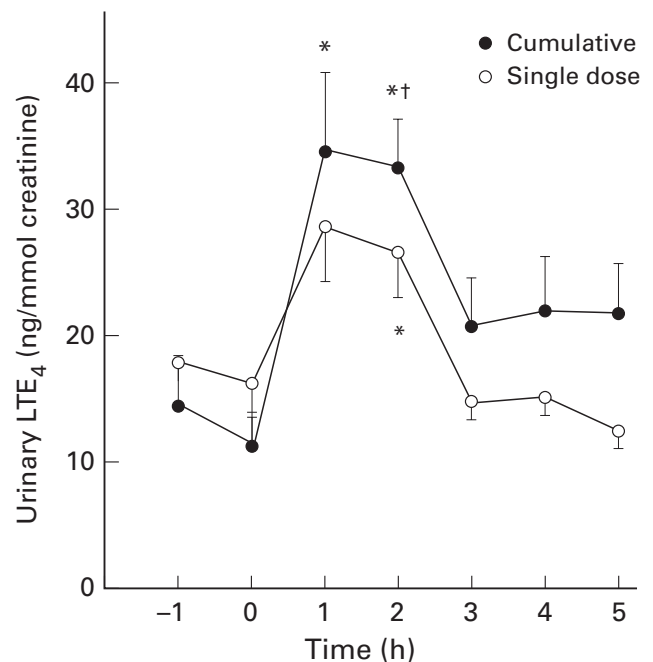


Fig. 1. Time-course of urinary LTE₄ concentrations in hourly collected samples before and after cumulative (filled circles) and single dose (open circles) allergen bronchoprovocation in seven atopic asthmatics. *Significant ($P < 0.05$) increase from the baseline value ($t = 0$). †denotes significant ($P < 0.05$) difference between values after single dose and cumulative challenge.

also a non-significant trend for a slightly prolonged elevation in urinary LTE₄ following the cumulative challenge, but the significant increase above baseline was for both challenges over within 3 h after the peak drop in FEV₁ (Fig. 1).

The extent of urinary LTE₄ excretion following the two sessions was compared by calculating the net increase (post-challenge minus prechallenge levels) during the three 3 h after the challenge. The net increase in urinary LTE₄ was more than twofold higher after the cumulative challenge (Fig. 2).

Discussion

The study showed that cumulative challenge with allergen resulted in significantly higher post-challenge levels of urinary LTE₄ in comparison with single dose administration of the same amount of allergen, despite the fact that the same degree of bronchoconstriction was obtained at both sessions. Using cumulative bronchoprovocations we have previously demonstrated data supporting dose-dependent urinary excretion of LTE₄ in response to allergen [7] as well as to inhaled lysine-aspirin in NSAID-intolerant asthmatic subjects [11]. The finding in this study, points to an additional mechanism where repetitive stimulation somehow

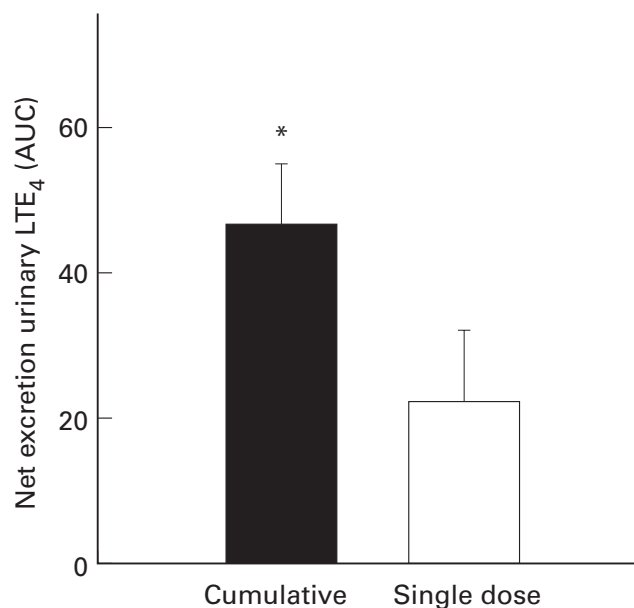


Fig. 2. Net excretion of urinary LTE₄ (post-challenge minus prechallenge values) in response to single dose or cumulative allergen bronchoprovocation in seven subjects, calculated as AUC during the first three hours after either challenge. *Significant ($P < 0.05$) difference between the two procedures.

leads to enhanced leukotriene production. The mechanism behind the observed effect is not known, but might involve some priming of inflammatory cells by repetitive stimulation [20]. Although not investigated in this study, it is conceivable that the production of other preformed or newly synthesized mediators is enhanced by cumulative dosing of allergen.

The results clearly have practical implications for studies where allergen-induced excretion of LTE₄ is evaluated, for example when the efficacy of drugs which inhibit leukotriene formation are to be assessed *in vivo*. Our findings indicate that when urinary LTE₄ is the endpoint, cumulative dosing of allergen may provide a stronger signal to be inhibited than that evoked by single dose challenge. When the fall in FEV₁ is the primary endpoint, the choice of challenge procedure apparently makes no difference. The similar degree of bronchoconstriction at the two sessions may seem surprising, but is in line with several other studies suggesting no direct correlation between drop in FEV₁ and urinary LTE₄ levels [4,7,10]. Sladek *et al.*, however, described such a correlation [5]. One reason for the lack of direct correlation is obviously that other mediators, such as histamine, contribute to the bronchoconstriction [13].

The peak increase in urinary LTE₄ was detected in the urine portion collected about 1 h after the maximal fall in FEV₁, irrespective of whether cumulative or single dose challenge was used. This finding is hardly surprising in view

of published studies of the relatively rapid renal elimination of cysteinyl-leukotrienes after intravenous injection [21,22] or inhalation [7,23], but emphasizes that the peak excretion of LTE₄ following a burst of pulmonary formation of cysteinyl-leukotrienes is fairly short-lasting. As a consequence, a short-lasting increase in urinary LTE₄ may be diluted and neglected if urine is collected at too long time intervals. It is, for example, of interest in this context that Friedman *et al.* [15] did not find any significant post-challenge increase in LTE₄ when urine was collected in 3-h periods after single dose allergen inhalation. Moreover, when calculating the mean value of the 3 hourly samples collected after the single dose allergen challenge in this particular study, there was no longer a significant increase above the prechallenge baseline (23.3 ± 2.3 after and 17.0 ± 2.7 before; $P = 0.15$), although it was possible to detect a significant doubling in urinary LTE₄ when the hourly collected samples were compared (Fig. 1).

In conclusion, the results of our study indicate that the magnitude of urinary excretion of LTE₄, in addition to the total allergen dose administered, is dependent on whether or not cumulative challenge is performed. Furthermore, because of the rapid excretion of LTE₄ into the urine, a transient increase in LTE₄ may only be detected if urine is collected often, for example on an hourly basis.

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