Circadian Characteristics of Urinary Leukotriene E4 in Healthy Subjects and Nocturnal Asthmatic Patients*

Keizo Kurokawa, MD; Hiroshi Tanaka, MD; Shintaro Tanaka, MD; and Shosaku Abe, MD

Study objectives: Circadian rhythmicity of cysteinyl leukotrienes (LTs) and thromboxane (TX)-A2 in healthy subjects and nocturnal asthmatic patients remains a subject of controversy. The aim of this study was to investigate the contribution of these mediators to the pathogenesis of nocturnal asthma.

Methods: We measured peak expiratory flow rate, urinary concentration of LTE4, 11-dehydro-TXB2, and creatinine eight times every 3 h in three groups: healthy control subjects (n = 5, group A), nocturnal asthmatic patients (n = 9, group B), and nonnocturnal asthmatic subjects (n = 9, group C). To evaluate the reproducibility of the measurement of urinary LTE4, we measured urinary LTE4 in group A for 3 separate days.

Results: The urinary LTE4 concentrations from 3 to 6 AM were significantly (p < 0.05) higher than from 3 to 6 PM in both group A and group B, but not in group C. The mean levels of LTE4 in group B and group C were significantly higher (p < 0.05) than those in group A. In group B, another small peak was observed from 6 to 9 PM. No significant day-to-day variation was observed in group A. Urinary 11-dehydro-TXB2 values from 3 to 6 AM were significantly (p < 0.001) higher than those levels from 3 to 6 PM in all groups, and the mean levels in group B and group C were significantly higher than those in group A (p < 0.05).

Conclusions: Circadian rhythmicity of urinary LTE4 with a morning peak was found in healthy control subjects and nocturnal asthmatic subjects, but not in nonnocturnal asthmatic patients. It was suggested that cysteinyl LTs rather than TXA2 might contribute to the nocturnal worsening of asthma.

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Key words: circadian rhythm; leukotriene; nocturnal asthma; peak expiratory flow; thromboxane

Abbreviations: %FEV1 = baseline percentage of FEV1; LT = leukotriene; PEFR = peak expiratory flow rate; TX = thromboxane

Cysteinyl leukotrienes (LTs) and thromboxane (TX)A2 are metabolites of arachidonic acid,1 and they have bronchoconstrictive effects that are ≥ 2,000 times that of histamine.1–3 Therefore, both mediators are thought to have a critical role in asthmatic patients. In the lung, LTC4 is rapidly converted to LTD4 and further to LTE4. After IV infusion of LTC4 or inhalation of LTD4, a constant proportion (2 to 6%) of these LTs was excreted in urine in the form of LTE4.4,5 Urinary LTE4 excretion rates are known to increase during severe asthma attacks,6 and to decrease during 5-lipoxygenase inhibitor treatment.7 Urinary LTE4 is therefore an appropriate marker of the systemic production of cysteinyl LTs.8,9 TXA2 is also a potent bronchoconstrictor, and there were evidences of elevation in TXA2 levels on asthma exacerbation or after allergen challenge.10–12 Reliable measurement of TXA2 in plasma is difficult because of the rapid conversion and resulting artifacts of this substance during blood sampling.1,3,13,14 It is excreted into urine as a stable form of 11-dehydro-TXB2.13

Nocturnal exacerbation is a very common event in patients with asthma,15,16 although its exact cause is far from clear. In general, catecholamines, corticosteroids, vagal tone, inflammatory mediators, mucociliary clearance, and β2-agonist responsiveness have all been shown to strengthen the potential for nocturnal exacerbation.17–19 Urinary adrenaline or noradrenaline, and plasma histamine have been correlated to morning dips in peak expiratory flow rate.
Nocturnal asthma is associated with a significant increase of both eosinophils in BAL fluid and eosinophils and macrophages in the alveolar tissue at 4 AM. These inflammatory cells are known to be capable of generating LTs and TXA₂.¹

Asano et al measured urinary LTE₄ values from patients with asthma and control subjects in 6-h intervals: 12 noon to 6 PM, 6 PM to 12 midnight, 12 midnight to 6 AM, and 6 AM to 12 noon. They reported that overall urinary LTE₄ levels were higher in asthmatics compared to control subjects, but no circadian variation in either group was appreciated. However, Bellia et al measured urinary LTE₄ levels in control subjects, and asthmatics subjects with and without nocturnal worsening, and found no difference in urinary LTE₄ levels between the control subjects and nonnocturnal asthmatics. The LTE₄ levels were significantly higher in the nocturnal asthmatic group at night compared to the other two groups, and a significant linear correlation was demonstrated between the morning dip in PEFR and the log of the urinary LTE₄ level. In spite of several studies of circadian variations focusing on urinary LTE₄ and 2,3-dinor-TXA₂,¹¹ circadian rhythmicity of cysteinyl-LTs in healthy subject and asthmatic patient remains a subject of controversy.²² We hypothesized that daily variations of arachidonic acid metabolites, especially LTs, might be associated with nocturnal exacerbation of asthma. In this study, we measured circadian changes of PEFRs, urinary LTE₄, and 11-dehydro-TXB₂ in healthy subjects, and patients with and without nocturnal worsening, and assessed a contribution of these arachidonic acid metabolites to the pathogenesis of nocturnal asthma exacerbation.

MATERIALS AND METHODS

Subjects

All subjects were recruited from Sapporo Medical University Hospital. As a normal control group, five healthy volunteers (four men and one woman, aged 31 to 40 years) were selected because of their lack of family and personal history of allergy or respiratory diseases (group A). Their baseline percentage of FEV₁ (%FEV₁) was 99.5%. Nine nocturnal asthmatic patients (group B: six men and three women, aged 21 to 77 years) and nine nonnocturnal asthma subjects (group C: four men and five women, aged 17 to 77 years) were chosen at random from a larger group of nonaspirin-sensitive asthmatic patients. Asthma was defined using the criteria of the American Thoracic Society and they had no positive reactions to antigen-specific serum IgE in 23 common antigens tested by a commercial kit (CAP RAST System; Pharmacia Diagnostics; Uppsala, Sweden), and patients were classified as atopic when they had at least one or more positive reactions to antigen-specific serum IgE. According to these criteria, 11 patients were nonatopic and 7 patients were atopic in groups B and C.

Study Design

All subjects were admitted to Sapporo Medical University Hospital during the study. Theophylline and inhaled β₂-agonist treatments were stopped 24 h before the study began. The subjects refrained from exercise and were kept at rest during the 24 h of the study. Urine sampling began at noon on the first study day. Urine was collected for determination of creatinine, LTE₄, and 11-dehydro-TXB₂. Urinary sample collection and PEFR measurements were performed every 3 h (at 3 PM, 6 PM, 9 PM, midnight, 3 AM, 6 AM, 9 PM, and 12 midnight). PEFRs were measured using a flowmeter (Mini-Wright Peak Flowmeter; Clement Clarke International; Harlow, Essex, UK). To evaluate the reproducibility of the measurement of urinary LTE₄, we measured urinary LTE₄ for 3 separate days in normal control subjects (group A).

Measurement of Urinary LTE₄

Approximately 30 mL of urine was collected from each patient in a polystyrene container. Immediately after collection, 4 mL of solution (ethyl acetate: methanol, 2:1) was added to each 1 mL of urine specimen to eliminate proteins; the specimens were then frozen at −80°C until the assays were performed for urinary creatinine and LTE₄. This method has been previously reported.²³,2⁶ Briefly, after a reverse-phase, high-pressure liquid chromatography gradient system equipped with a Nova-Pak C₁₈ column (Waters Associates; Milford, MA), LTE₄ was measured (LT³[H] radioimmunoassay kit; DuPont New England Nuclear Research; Boston, MA). The measurements were corrected by the creatinine content of urine; levels were expressed as picograms per milligram of creatinine. The recovery rate of tritiated LTE₄ was from 50 to 60%. The innerassay and interassay coefficients of variation were 11% and 10%, respectively.

Measurement of Urinary 11-dehydro-TXB₂

Indomethacin was added to the urine samples and stored at −80°C. We have previously reported the method of measurement of urinary 11-dehydro-TXB₂.²⁵,²⁶ After deproteinization and defatting, the samples were applied to an Si minicolumn BOND ELUT SI (Varian; Harbor City, CA) and consequently fractionated by eluent 1 (chloroform: acetic acid, 100:0.5), eluent 2 (acetonitrile: chloroform: acetic acid, 10:90:0.5), and eluent 3 (acetonitrile: chloroform: acetic acid, 20:80:0.5). The fraction obtained by eluent 3 was evaporated using N₂ gas and reconstructed by the buffer of the 11-dehydro-TXB₂ [¹²⁵I] radio-
Statistical Analysis

Results were expressed as mean ± SE. Mann-Whitney U test and χ² test were used for analysis of patient backgrounds, mean values of PEFRs, and levels of urinary arachidonic acid metabolites. Day-to-day variations of urinary LTE₄ excretion rate in group A were assessed by repeated-measures analysis of variance. Comparison of urinary LTE₄ levels from 6 AM to 6 PM was performed using Mann-Whitney U test. All p values < 0.05 were considered statistically significant.

RESULTS

No patients needed rescue medications during this study. PEFRs obtained from the asthma diary were not different from before the study and during the study. The mean value of PEFRs in atopic asthmatics (n = 7) was not different from that in nonatopic asthmatics (n = 11). The mean levels of urinary metabolites of the eight measurements were not significantly different between atopic and nonatopic asthmatics in urinary LTE₄ and 11-dehydro-TXB₂ levels.

PEFR

PEFR values at 6 AM were lower than values at 6 PM, indicating circadian rhythm in group B (407 ± 14 L/min vs 315 ± 19 L/min, p < 0.001), but not in groups A and C (Fig 1). The means of group B and group C were significantly lower than those of group A (p < 0.05; Table 2).

LTE₄ Excretion

The mean levels of LTE₄ in group B and group C were significantly higher (p < 0.05) than those in

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### Table 1—Subject Characteristics*

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Smoking</th>
<th>Atopy</th>
<th>Duration of Asthma, yr</th>
<th>Peripheral Blood Eosinophil, μL</th>
<th>Baseline FEV₁, L</th>
<th>Baseline % FEV₁ Predicted</th>
<th>Medications</th>
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</table>

*NS = nonsmoker; S = smoker; β = inhaled β₂-agonist as required; T = theophylline; M = male; F = female.
†p < 0.05.
‡p < 0.01.
§p < 0.005 compared with group A.
group A (Table 2). In group B, another small peak was observed in from 6 to 9 PM (Fig 1, middle). The comparison of urinary LTE$_4$ levels from 3 to 6 PM and from 3 to 6 AM in each group is shown in Figure 2. There were significant differences in group A (113 ± 35 pg/mg creatinine vs 168 ± 54 pg/mg creatinine, p = 0.046) and group B (182 ± 60 pg/mg creatinine vs 252 ± 121 pg/mg creatinine, p = 0.021). In all patients in group B (n = 9), urinary LTE$_4$ values from 3 to 6 AM were higher than those from 3 to 6 PM. The diurnal variation of mean urinary LTE$_4$ in group A for 3 separate days is shown in Figure 3. No significant day-to-day variation was observed in five subjects in group A.

11-dehydro-TXB$_2$ Excretion

The mean levels of 11-dehydro-TXB$_2$ in group B and group C were significantly higher (p < 0.05) than in group A (Table 2). The comparison of urinary 11-dehydro-TXB$_2$ levels from 3 to 6 PM and from 3 to 6 AM showed significant (p < 0.01) difference in all groups (523 ± 29 pg/mg creatinine vs 748 ± 57 pg/mg creatinine in group A, 592 ± 60 pg/mg creatinine vs 1140 ± 108 pg/mg creatinine in group B, and 762 ± 65 pg/mg creatinine vs 1,193 ± 94 pg/mg creatinine in group C). Circadian rhythmicities in urinary 11-dehydro-TXB$_2$ levels were detected in all groups with morning acrophases, and the mean levels in group B and group C were significantly higher than those in group A (p < 0.05; Table 2, Fig 1).

**Discussion**

We found a circadian rhythmicity of urinary LTE$_4$ levels with morning acrophase in normal subjects. However, diurnal variations of urinary LTE$_4$ in normal subjects have been denied in previous reports. In our study, urinary samples were obtained every 3 h, but in the previous studies, urinary samples were obtained every 6 to 12 h. LTE$_4$ appeared in urine within the first 1 to 4 h after IV infusion or inhalation of cysteinyl-LTs; therefore, 3-h urine sampling would reflect the detail changes of LTs that occurred over the 3 h prior to sampling. In fact, when we recalculated the mean urinary LTE$_4$ levels of the normal control subjects every 6 h, there was no circadian rhythmicity. This may be one of the major reasons for the different results between the previous studies and ours. We also evaluated the reproducibility of our measurement for urinary LTE$_4$ in healthy subjects, and found no significant day-to-day variation for 3 separate days. There has been little study on day-to-day variability of urinary LTE$_4$ excretion rate. Asano et al. also reported that no significant day-to-day variations of

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**Figure 1.** Diurnal variation of the mean values of PEFRs, urinary LTE$_4$ and 11-dehydro-TXB$_2$ in group A (control, top), group B (nocturnal asthma, middle), and group C (nonnocturnal asthma, bottom). Samples were obtained every 3 h for 24 h, beginning at 12 noon.
urinary LTE_4 levels existed for 4 days in both normal subjects and asthmatic patients. To our knowledge, this is the first report that urinary LTE_4 excretion had regular circadian rhythmicity with morning acrophase in normal subjects.

Urinary LTE_4 levels had a circadian variation in patients with nocturnal asthma; however, this was not the case in patients with nonnocturnal asthma, suggesting that LTs might be important in the pathogenesis of nocturnal asthma. Circadian rhythm of urinary 11-dehydro-TXB_2 with morning acrophase was observed in both nocturnal and nonnocturnal asthmatic patients. These results suggested that cysteinyl LTs might play a more important role in the nocturnal worsening of asthma than TXA_2. It has been reported that an inverse correlation was found between urinary LTE_4 and FEV_1, but no correlation was observed between urinary 11-dehydro-TXB_2 excretion and FEV_1 in asthmatic patients. Although urinary 11-dehydro-TXB_2 excretion has been shown to increase during severe asthmatic exacerbations, TXA_2 might play only a minor role in nocturnal asthmatic exacerbation.

Our data also demonstrated a small peak of LTE_4 in urine from 6 to 9 PM in patients with nocturnal asthma, and PEFR progressively decreased in this period. In general, there were significant increases of eosinophils in BAL fluid and eosinophils and macrophages in the alveolar tissue of nocturnal asthmatics at 4 AM, which would be connected with the morning dip. However, Oosterhoff et al suggested that increased cellular activation during the daytime (4 PM) occurred in subjects with nocturnal asthma compared with normal control subjects and nonnocturnal asthmatics. Fitzpatrick et al also reported that serum eosinophil cationic protein levels decreased at night (2 to 4 AM) compared with the daytime in nocturnal asthma. Reinberg et al reported that in asthmatic patients, an injection of methylprednisolone at 3 PM had more improvement on PEFR than an injection at 3 AM. Furthermore, Beam et al studied the timing of a single dose of prednisolone (8 AM, 3 PM, or 8 PM) in subjects with nocturnal asthma, and patients experienced the largest improvement in pulmonary function at the 3 PM administration. Airway inflammation from late afternoon to evening (6 to 9 PM) might be important for the mechanism of nocturnal worsening. We specu-

Table 2—Means of PEFRs and Urinary Arachidonic Acid Metabolites

<table>
<thead>
<tr>
<th>Groups</th>
<th>PEFR, L/min</th>
<th>LTE_4, pg/mg Creatinine</th>
<th>11-dehydro-TXB_2, pg/mg Creatinine</th>
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<tbody>
<tr>
<td>Control (group A)</td>
<td>567.2 ± 26.1</td>
<td>137.0 ± 33.1</td>
<td>646.5 ± 85.3</td>
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<td>Nocturnal asthma (group B)</td>
<td>368.8 ± 78.2</td>
<td>220.0 ± 26.0†</td>
<td>956.0 ± 141.8†</td>
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<tr>
<td>Nonnocturnal asthma (group C)</td>
<td>382.5 ± 23.4</td>
<td>208.6 ± 22.3†</td>
<td>945.8 ± 137.6†</td>
</tr>
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</table>

*Data are presented as mean ± SE.
†p < 0.05 compared with group A.
lated that inflammatory cells would be activated and LT excretions might begin from 6 to 9 PM, and these activated inflammatory cells would migrate and accumulate to airways and trigger the nocturnal worsening. It was suggested that asthma treatment with corticosteroids in the late afternoon and evening might be effective in preventing the subsequent PEFR depression seen in nocturnal asthmatics.

We could not elucidate the mechanisms underlying the difference in circadian rhythms of urinary LTE4 between nocturnal and nonnocturnal asthmatic patients. There were no differences in asthma therapy between the two groups. Therefore, the reason why rhythmicity of PEFR and urinary LTE4 levels was small in nonnocturnal asthmatic patients would not be due to their treatment modality. From our results, although urinary 11-dehydro-TXB2 levels had a similar circadian rhythm to normal control subjects and nocturnal asthmatics, PEFRs levels significantly decreased and urinary LTE4 values significantly increased as compared with normal subjects. Therefore, it was speculated that increased LTs due to asthma inflammation, including eosinophils involvement, without circadian rhythmicity might lead to decreased PEFRs without circadian rhythmicity. One of the possible reasons for the different LTE4 circadian rhythms might be interaction among other eicosanoids. No significant differences were detected in circadian variations of urinary 11-dehydro-TXB2 between the two groups; therefore, the effects of TXA2 would not explain the difference. It was reported33 that prostaglandin E2 prevented aspirin-induced asthma by inhibiting the release of cysteinyl-LTs. Plasma prostaglandin E2 levels had a circadian rhythm with an acrophase at 5:04 AM in patients with nocturnal asthma.34 Further studies on interactions between inflammatory mediators and their inhibitors are required.

To our knowledge, there has been no report as to whether smoking affects levels of urinary LTE4. We have measured urinary LTE4 and 11-dehydro-TXB2 levels in > 300 asthmatic patients (H. Tanaka, MD; unpublished data; December 2000). These data revealed no smoking effect on urinary LTE4 excretion. However, enhanced TX biosynthesis was reported in COPD patients who smoke.35 We recalculated the mean levels of urinary 11-dehydro-TXB2 in each group after excluding smokers. The mean value of urinary 11-dehydro-TXB2 changed from 646 to 550 pg/mg creatinine in control subjects, from 956 to 802 pg/mg creatinine in nocturnal asthmatic patients, and from 945 to 912 pg/mg creatinine in nonnocturnal asthmatics. In all groups, the mean levels of urinary 11-dehydro-TXB2 had a tendency to decrease when the smokers were excluded, but there was no change in the circadian rhythmicity with morning acrophase. Normal volunteers were younger than asthmatic patients in this study. To our knowledge, there have been no reports of the effect of aging on urinary secretion of LTE4 and 11-dehydro-TXB2. According to our unpublished data of nonsmoking asthmatics, there were no aging effects on urinary LTE4 or 11-dehydro-TXB2 levels. Therefore, aging and smoking can be judged to have had no effect in our circadian results.

In conclusion, the present study demonstrated circadian rhythmicity of urinary LTE4 in healthy subjects and nocturnal asthmatic patients with an acrophase in the morning, but this was not the case for nonnocturnal asthmatic subjects. The mean urinary LTE4 concentrations in patients with both nocturnal and nonnocturnal asthma were significantly higher in control subjects. In nocturnal asthmatic subjects, there were two small peaks in the late afternoon and morning; the former might trigger airway inflammation in the late afternoon, and the latter may induce morning exacerbation. A similar circadian rhythm with morning acrophase in urinary 11-dehydro-TXB2 levels was found in all three groups, but the mean level in nocturnal and nonnocturnal asthmatics was higher than that in control subjects. These findings suggested that LTs rather than TXA2 might contribute to the circadian rhythmicity of nocturnal asthma.

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