Aspirin-Intolerant Asthma (AIA) Assessment Using the Urinary Biomarkers, Leukotriene E4 (LTE4) and Prostaglandin D2 (PGD2) Metabolites

Noritaka Higashi1, Masami Taniguchi1, Haruhisa Mita1, Hiromichi Yamaguchi1, Emiko Ono1 and Kazuo Akiyama1

ABSTRACT
The clinical syndrome of aspirin-intolerant asthma (AIA) is characterized by aspirin/nonsteroidal anti-inflammatory drug intolerance, bronchial asthma, and chronic rhinosinusitis with nasal polyposis. AIA reactions are evidently triggered by pharmacological effect of cyclooxygenase-1 inhibitors. Urine sampling is a non-invasive research tool for time-course measurements in clinical investigations. The urinary stable metabolite concentration of arachidonic acid products provides a time-integrated estimate of the production of the parent compounds in vivo. AIA patients exhibit significantly higher urinary concentrations of leukotriene E4 (LTE4) and 1,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM), a newly identified metabolite of PGD2, at baseline. This finding suggests the possibility that increased mast cell activation is involved in the pathophysiology of AIA even in a clinically stable condition. In addition, lower urinary concentrations of primary prostaglandin E2 and 15-epimer of lipoxin A4 at baseline in the AIA patients suggest that the impaired anti-inflammatory elements may also contribute to the severe clinical outcome of AIA. During the AIA reaction, the urinary concentrations of LTE4 and PGD2 metabolites, including tetranor-PGDM significantly and correlatively increase. It is considered that mast cell activation probably is a pathophysiologic hallmark of AIA. However, despite the fact that cyclooxygenase-1 is the dominant in vivo PGD2 biosynthetic pathway, the precise mechanism underlying the PGD2 overproduction resulting from the pharmacological effect of cyclooxygenase-1 inhibitors in AIA remains unknown. A comprehensive analysis of the urinary concentration of inflammatory mediators may afford a new research target in elucidating the pathophysiology of AIA.

KEY WORDS
aspirin, asthma, biomarker, cysteinyl-leukotriene, mast cell, prostaglandin D2, urine

ABBREVIATIONS
AIA, Aspirin-intolerant asthma; NSAID, Nonsteroidal anti-inflammatory drug; CRSwNP, Chronic rhinosinusitis with nasal polyps; COX, Cyclooxygenase; CysLT, Cysteinyl-leukotriene; PGD2, Prostaglandin D2; LTE4, Leukotriene E4; HPLC, High-performance liquid chromatography; ATA, Aspirin-tolerant asthma; 15-epi-LXA4, 15-Epimer of lipoxin A4; IgE, Immunoglobulin E; LXA4, Lipoxin A4; 9α,11β-PGF2, 9α,11β-Prostaglandin F2; 2,3-Dinor-9α,11β-PGF2; 2,3-Dinor-9α,11β-prostaglandin F2; Tetranor-PGDM, 11,15-Dinor-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid; EIA, Enzyme immunoassay; ent-PGF2α, Prostaglandin F2α enantiomer;
INTRODUCTION
The clinical syndrome of aspirin-intolerant asthma (AIA) is characterized by aspirin/nonsteroidal anti-inflammatory drug (NSAID) intolerance, bronchial asthma and chronic rhinosinusitis with nasal polyposis (CRSwNP). Aspirin/NSAID-induced asthma reactions are triggered by pharmacological effect of cyclooxygenase-1 (COX-1) inhibitors, whereas COX-2-specific inhibitors (coxibs) are tolerated in the vast majority of cases. Several clinicoepidemiologic studies have demonstrated that AIA is one of the common risk factors for the development of refractory asthma. Previous in vitro studies demonstrated the release of chemical mediators from leukocytes in AIA patients and these in vitro tests may be applicable to a diagnosis of aspirin/NSAIDs intolerance. However, there is no experimental evidence which directly supports this hypothesis. That is, there is neither an appropriate in vitro test to diagnose AIA nor an animal model to help fully elucidate the pathogenesis of AIA. Thus, biological fluid samples from AIA subjects are the only research tools available. Urine samples constitute a non-invasive research tool for time-course measurements in the clinical setting, although such data does not provide any information on the sites of the production. The urinary concentration of chemical mediator metabolites is remarkably

<table>
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<th>Table 1 Inflammatory mediators in biological samples</th>
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<tr>
<td>invasive</td>
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<tr>
<td>BALF</td>
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<tr>
<td>unmetabolized compounds</td>
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<td>local production</td>
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<td>instantaneous measure of endogenous production of the compounds</td>
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<td>ext. high levels</td>
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Abbreviations: BALF, bronchoalveolar lavage fluid; ELF, epithelial lining fluid; EBC, exhaled breath condensate; ext., extremely.

![Fig. 1 Arachidonic acid cascade (Adapted from Yamaguchi et al.].
Urinary LTE4 and PGD2 Biomarkers in AIA

Changes in the urinary LTE4 concentrations following endoscopic sinus surgery (ESS). Horizontal bars indicate medians. Patients with aspirin-intolerant asthma (AIA, n = 19) and patients with aspirin tolerant asthma (ATA, n = 8) are denoted by circles and squares, respectively. Additional urinary data (12 AIA patients, closed circles) were included in the original figure published previously. The asterisks (*, **) indicate p < 0.05 and p < 0.01, respectively, in comparison with baseline values (pre-ESS).

Urinary Leukotriene (LT) E4 in AIA at Baseline

Since the leukotriene (LT) C4 is easily metabolized in the lungs and/or the liver, and then disappears into the body fluid, LTE4 is the predominant metabolite among the CysLTs of defined structure. It is reported that 4-13% of the intravenous or inhaled dose of LTC4 is excreted in the urine. Arachidonic acid metabolites are present in extremely small quantities in biological fluids (on an order ranging from pg/ml to ng/ml). When assayed by enzyme immunoassay (EIA), biological samples should be purified in order to eliminate any interfering substances. Thin-layer chromatography has been commonly employed to remove such interfering substances. We believe that purification using high-performance liquid chromatography (HPLC) is also convenient and provides a suitable quantification procedure. Recently it has been emphasized that additional chromatographic steps are required for obtaining reliable data on the urinary LTE4 concentrations. There is increasing evidence that the AIA group exhibits a significantly higher urinary LTE4 excretion level at baseline than the aspirin-tolerant asthma (ATA) group, even in a clinically stable condition. Of late it appears that the baseline urinary LTE4 concentrations in the AIA group are on a decline compared with original data by Christie et al. and Kumlin et al., perhaps because of a stabilization of asthma symptoms by inhaled corticosteroids. Considering the distinct evidence from several immunohistochemical studies, LTC4 producing cells such as eosinophils and mast cells seem to contribute to increased baseline concentrations of urinary LTE4 in subjects with AIA. Interestingly, we have demonstrated that a severe ATA group with chronic rhinosinusitis with nasal polyposis (CRSwNP) also exhibited a significantly higher urinary LTE4 concentration at baseline. That is, CysLT overproduction is associated with the clinical features of severe asthma with CRSwNP, that is, the so-called “aspirin triad” in AIA. Taken together with the evidence that there is a close relationship between CRSwNP and CysLT overproduction in asthmatic subjects, we have proposed the concept of “hyper-leukotrienuria”. The preliminary data indicates that aspirin intolerance seldom develops in pa-
Evidence of clinical improvement of asthma symptoms after endoscopic sinus surgery (ESS). Horizontal bars indicate means. The asterisk (*) indicates $p < 0.05$ in comparison with baseline values (pre-ESS, $n = 9$).

![Graph showing ER visits and Ach-PC20](image)

Table 2  Analytical significance of urinary eicosanoid concentrations

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<tr>
<th>Urinary eicosanoids</th>
<th>Metabolites compounds</th>
<th>Unmetabolites compounds</th>
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<tr>
<td></td>
<td>LTE₄</td>
<td>D-ring PGDM</td>
</tr>
<tr>
<td>Derived from</td>
<td>5-LO</td>
<td>COX (COX-1 dominant)</td>
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<tr>
<td>As an index of</td>
<td>whole body production</td>
<td>local kidney production</td>
</tr>
<tr>
<td></td>
<td>LTE₂</td>
<td>PGD₂</td>
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Abbreviations: LTE₄, leukotriene E₄; 5-LO, 5-lipoxygenase; PGDM, prostaglandin D₂ metabolite; F-ring PGDM, 9α,11β-prostaglandin F₂ and 2,3-Dinor-9α,11β-prostaglandin F₂; D-ring PGDM, tetranor-PGDM; PGE₂, prostaglandin E₂; PGEM, 13,14-dihydro-15keto-prostaglandin E₂; tetranor-PGEM, 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid; COX, cyclooxygenase; ent-PGF₂αβ, prostaglandin F₂α enantiomer.

The original table has been published previously with some modification.25

... with ATA, even with hyper-leukotrienuria, throughout the course of a 5-year follow-up period (unpublished data). This clinical finding leads us to the hypothesis that CysLT overproduction in vivo does not promote aspirin intolerance by itself. Interestingly, it was demonstrated for the first time that there is a significant decrease in the urinary LTE₄ concentrations after endoscopic sinus surgery in both the AIA and ATA groups. We carried out further investigation in 12 additional AIA patients, and found further evidence which suggests that CRSwNP is involved in CysLT overproduction in asthmatic subjects, as shown in Figure 2. Furthermore, we have preliminarily determined the clinical improvement in asthma-related emergency room visits and bronchial hyperresponsiveness after endoscopic sinus surgery. Unexpectedly, sinus surgery resulted in significantly fewer asthma-related emergency room visits. In addition, there were significant increases in the Ach-PC₂₀ values after endoscopic sinus surgery. (unpublished data, Fig. 3) These data typically support the concept of “one airway, one disease”.36 Recent research suggests a close relationship between LT biosynthesis and vascular events such as arteriosclerosis.37,38 We have reported significantly increased urinary LTE₄ concentrations in patients with acute exacerbated vasculitides.39 It is also reported that the urinary LTE₄ concentration is slightly increased in current smokers40 and obese subjects,41 suggesting that the urinary LTE₄ concentration may be useful as a non-invasive biomarker of oxidative tissue inflammation and related pathophysiologic events.42

**URINARY PGs CONCENTRATIONS IN AIA AT BASELINE**

Recently we reported that the urinary PGE₂ concentrations at baseline in the AIA group are significantly lower than the ATA group.25 Since PGE₂ in urine is an unmetabolized compound, the primary PGE₂ concentrations in urine have been considered to predominantly reflect local renal production43 (Table 2). Interestingly, lower spontaneous production of PGE₂ has been reported in epithelial cells from nasal polyps44 and sinonasal tissue45 in subjects with AIA,
which is associated with diminished COX-2 expression in these tissues.\textsuperscript{46,47} Inhaled PGE\textsubscript{2} protects against both aspirin-induced bronchoconstriction and the massive release of urinary LTE\textsubscript{4},\textsuperscript{48} so a critical deficiency in PGE\textsubscript{2} “braking” has been postulated as one possible mechanism for the AIA reaction. Similarly we have demonstrated a decreased urinary concentration of 15-epimer of lipoxin A\textsubscript{4} (15-epi-LXA\textsubscript{4}),\textsuperscript{49} which is also produced by cell-to-cell interaction involving acetylated COX-2 and 5-lipoxygenase.\textsuperscript{50} In allergic airway inflammation, not only LXA\textsubscript{4} but also 15-epi-LXA\textsubscript{4} block both bronchial hyperresponsiveness and pulmonary inflammation induced by eosinophils via the LXA\textsubscript{4} receptor, leading to decreases in the numbers of eosinophils and T-lymphocytes and decreases in the concentrations of interleukin-5, interleukin-13, eotaxin, immunoglobulin E (IgE), PGs, and CysLTs.\textsuperscript{51} Thus, it is a plausible explanation that the low COX-2 expression \textit{in vivo} may result in the lower urinary concentrations of anti-inflammatory PGE\textsubscript{2} and 15-epi-LXA\textsubscript{4} at baseline in the AIA group, suggesting the deficiency of additional anti-inflammatory elements in AIA. Since the AIA subjects excreted significantly higher urinary LTE\textsubscript{4} concentration at baseline even in a clinically stable condition,\textsuperscript{23-29} as described above, an imbalance between the local production of pro-inflammatory CysLTs and anti-inflammatory 15-epi-LXA\textsubscript{4} and PGE\textsubscript{2} at baseline, may play an important role in development of refractory asthma in AIA. Unexpectedly, urinary lipoxin A\textsubscript{4} (LXA\textsubscript{4}) was significantly lower than 15-epi-LXA\textsubscript{4} because 15-epi-LXA\textsubscript{4} shows a two-fold longer half-life \textit{in vivo},\textsuperscript{52} as calculated by conversion rate of 15-hydroxyprostaglandin dehydrogenase.

**URINARY CONCENTRATIONS OF PG METABOLITES IN AIA AT BASELINE**

The major urinary metabolites of PGE\textsubscript{2} and PGD\textsubscript{2} are shown in Table 2. Among LTC\textsubscript{4} producing cells such as eosinophils, basophils and mast cells, it is only the mast cells that produce significant quantities of PGD\textsubscript{2} in human.\textsuperscript{53} Although there is evidence of some PGD\textsubscript{2} formation by eosinophils, platelets, macrophages and certain T lymphocytes, the reported amounts are 100 to 1000 times lower than those produced during IgE dependent activation of mast cells.\textsuperscript{54-56} So the urinary PGD\textsubscript{2} metabolites are useful biomarkers of mast cell activation.\textsuperscript{55} Although it is indeed also true that tryptase in the biological fluid samples is a mast cell-specific biomarker, it is impossible to detect tryptase in urine. Furthermore, it was reported that serum tryptase concentration was increased in only 6% of the patients who developed anaphylaxis.\textsuperscript{57} Similarly, Bochenek \textit{et al.} demonstrated that there was no change in serum tryptase despite the five-fold increase in plasma 9\textalpha,11\beta-PGF\textsubscript{2} follow-
ing the early phase of allergen-induced airway obstruction.58 Thus, we concluded that the determination of the urinary PGD2 metabolites is more sensitive and practical than serum tryptase for monitoring mast cell activation.59 “F-ring” urinary PGD2 metabolites, 9α,11β-prostaglandin F2 (9α,11β-PGF2) and 2,3-dinor-9α,11β-prostaglandin F2 (2,3-dinor-9α, 11β-PGF2), has been frequently used in the clinical studies.25,55,60,61 Recently, Song et al. newly identified the most abundant “D-ring” PGD2 metabolite in urine, 11,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM).61 We also examined the urinary tetranor-PGDM concentration with a newly commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Our data demonstrated that the urinary tetranor-PGDM concentrations were 4.8-fold (median) higher than 2,3-dinor-9α,11β-PGF2 or 11.7-fold higher than 9α,11β-PGF2 at baseline.62 Interestingly, the urinary concentrations of tetranor-PGDM and LTE4 at baseline in the AIA group was significantly higher than the anaphylaxis group62 (Fig. 4). To our knowledge, this is the first report of a significantly higher baseline concentration of PGD2 metabolites in urine in AIA patients, except for patients with mastocytosis.63 Thus, the quantification of tetranor-PGDM may be an attractive strategy for further investigation into mast cell activation. This finding is consistent with the previous reports of higher baseline concentration of sputum PGD2,27 plasma 9α,11β-PGF2 and serum tryptase in the AIA patients.64 Furthermore, it was reported that there was a significant increase in the number of submucosal mast cell in the bronchial biopsy obtained from AIA versus ATA patients.65,66 It is important to note that the purification of urine by HPLC is crucial for the precise quantification of tetranor-PGDM. One limitation of this biomarker of tetranor-PGDM is that it is generally unstable when not stored at -80°C. By contrast, there is no significant difference in the major urinary PGE2 metabolites, 13,14-dihydro-15keto-PGE2 (PGEM) and 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid (tetranor-PGEM), at baseline between AIA and ATA groups,67 in spite of the fact that COX-2 contributes substantially to the biosynthesis of PGE268 and that lower COX-2 expression is one of the unique characteristics of AIA.46,47 Interestingly, aspirin provocation decreases the urinary concentrations of PGE2 metabolites in only the ATA group, not the AIA group.67

**LTE4 AND PG METABOLITES DURING ASPIRIN/NSAIDs-INTOLERANT ASTHMA REACTION**

During systemic aspirin provocation, a runny nose and nasal congestion (>90%) are the first symptoms, and then acute bronchoconstriction develops more than 30 min after the administration of threshold dose of aspirin. This aspirin-induced bronchoconstric-
Song et al. demonstrated that the urinary tetranor-PGDM concentrations were suppressed by inhibition with aspirin, but not by a selective inhibition of COX-2.61 Interestingly, Daham et al. recently demonstrated that the urinary tetranor-PGDM concentration remains unchanged in both the AIA and ATA groups following the administration of the selective COX-2 inhibitor celecoxib.68 Taken together, despite the fact that COX-1 is the dominant in vivo PGD2 biosynthesis pathway, the precise mechanism underlying the PGD2 overproduction through the pharmacological effect of COX-1 inhibitors in the AIA group remains unknown.

PG PRODUCTION VIA THE FREE RADICAL-MEDIATED "ISOPROSTANE PATHWAY"

Recently, it was reported that there is another pathway in which arachidonic acid is metabolized in vivo by a free radical-mediated mechanism to yield a series of PG-like compounds termed isoprostanes that is independent on the catalytic activity of the COX enzyme.80 In contrast with COX-derived PGs, which is an optically pure form, the radical-mediated peroxidation of arachidonic acid generates a racemic mixture of PGs (Fig. 5). Thus, the presence of the enantiomer to COX-derived PG indicates that the PG is generated via a free radical-mediated mechanism.81 The concentration of the PG enantiomer in urine is a reliable index of systemic isoprostane and lipid oxidation.81,82 In particular, quantification of the urinary PGF2α enantiomer (ent-PGF2α) constitutes a valuable tool for assessing oxidant stress in vivo.82 As judged by the urinary ent-PGF2α concentration, free radical-mediated PG generation is also involved in the pathophysiology of IgE-mediated anaphylaxis.25 Therefore, we hypothesized that a free radical-mediated mechanism might be also responsible for the PGD2 production which occurs during the AIA reaction. However, unexpectedly, there was no change in the urinary ent-PGF2α concentrations after aspirin provocation in the AIA group.25 It seems that PGD2 overproduction in vivo in the AIA group after the aspirin provocation test is independent of the isoprostane pathway. More interestingly, recent studies on PG biosynthesis have demonstrated that nitric oxide nitrosylates cytosolic phospholipase A2 (cPLA2)83 and COX-2,84 resulting in the activation of these two enzymes and an increase in PG synthesis. These findings suggest that oxidative stress induces the post-translational modification of enzymes associated with eicosanoid biosynthesis.85

CONCLUSION AND CLINICAL RELEVANCE

This review article focuses on clinical significance of urinary biomarkers in AIA. We have analyzed a variety of biological samples, including serum, saliva,24,86 sputum,27 exhaled breath condensate87,88 and bronchoalveolar lavage fluid87,89 to assess the pathophysiology of allergic diseases such as AIA. To our knowledge, the biomarker of urinary LTE4 is the only appropriately sensitive biomarker for the aspirin-induced asthma/sinus reaction. Christie et al.90 have reported that AIA subjects exhibit selective hyperresponsiveness to LTE4, but not to LTC4, relative to that seen in ATA subjects. Interestingly, P2Y12, the adenosine diphosphate receptor, is responsible for the LTE4-mediated activation and proliferation of mast cells, as well as amplification of allergic pulmonary inflammation.91,92 Taken these findings together, it is suggested that a mechanism underlying AIA is that LTE4-mediated signaling pathway may play an important role for the development of refractory asthma. In addition, new receptors for LTE4 have recently been discovered. Their roles in CysLT signaling and related diseases, in particular AIA, need to be elucidated.93,94 Single measurements of urinary LTE4 concentrations do not allow a demonstration of the cellular source and target organ of CysLT production. We preliminarily compared the urinary LTE4 concentrations in the same patients (n = 3) exhibiting the same extent of bronchoconstriction upon systemic challenge and inhalation challenge. The duration between the two different challenges was more than 2 weeks to avoid the refractory period in AIA. The extent of increase in the urinary LTE4 concentration after systemic aspirin challenge was 3 to 10-fold higher than aspirin inhalation challenge (data not shown). This finding suggests that the site of CysLT production is not only the bronchi and lungs. Therefore, further quantification of the urinary PGD2 metabolite concentrations are helping to elucidate how
mast cell activation is involved in the pathophysiology of AIA.\(^{24,25,28,64,95}\) However, the precise mechanisms underlying the PGD\(_2\) overproduction in the AIA group, which occurred despite the administration of a COX inhibitor, remains unknown. Paruchuri \textit{et al.}\(^{23}\) have reported that LTE\(_4\) activates human mast cells by a pathway that involves a cooperation between MK571-sensitive G protein coupled receptors (GPCRs) and peroxisome proliferator activated receptor \(\gamma\) (PPAR\(\gamma\)), a nuclear receptor for dietary lipids. LTE\(_4\) possesses a capacity for upregulating COX-2 expression and causing PGD\(_2\) generation.\(^{96}\) Furthermore, He \textit{et al.}\(^{85}\) have demonstrated that treatment of bone marrow-derived mast cells (BMMC) with PGD\(_2\) reduces the ability of BMMC to generate LTC\(_4\) upon calcium ionophore stimulation, but has little effect on LT\(_B4\) generation. This effect can be reproduced by a selective agonist of the DP2 receptor, 15R-methyl-PGD\(_2\) (15R-PGD\(_2\)). 15R-PGD\(_2\) exerts its suppressive effect via a reduction in intracellular glutathione (GSH), a mechanism that involves the conjugation of its non-enzymatic breakdown product with GSH. Quantification of the new biomarker PGD\(_2\) metabolite tetranor-PGDM provides a clinically useful tool for assessing mast cell activation \textit{in vivo}. Urine samples afford a most non-invasive research tool for time-course measurements in a clinical study, and a lipidomics approach using biological fluid samples such as urine will provide further clinical data targeting mast cell activation, not only in allergic diseases, but also infectious diseases and cancer.

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