

Profile of eicosanoid generation in aspirin-intolerant asthma and anaphylaxis assessed by new biomarkers

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Background: It has recently demonstrated that a free radical-mediated pathway generates prostaglandins (PGs) and the corresponding prostaglandin enantiomers (*ent*-PGs). Aspirin-intolerant asthma and anaphylaxis accompany PGD₂ overproduction, possibly associated with mast cell activation via the COX pathway. However, free radical-mediated PG generation in the pathophysiology of these diseases, which can be demonstrated by measuring urinary *ent*-PGF₂α, has not been reported.

Objectives: To evaluate the characteristic profile of eicosanoid generation via the COX and/or free radical-mediated pathway underlying aspirin-intolerant asthma and anaphylaxis.

Methods: A comparative group analysis consisted of asthma (n = 17) and anaphylaxis (n = 8, none with aspirin-induced anaphylaxis) cases. Urinary eicosanoid concentrations were quantified as follows: 2,3-dinor-9α,11β-PGF₂ by gas chromatography-mass spectrometry; leukotriene E₄, 9α,11β-PGF₂, and PGs by enzyme immunoassay.

Results: 2,3-Dinor-9α,11β-PGF₂ is a more predominant PGD₂ metabolite in urine than 9α,11β-PGF₂. At baseline, the aspirin-intolerant asthma group (n = 10) had significantly higher leukotriene E₄ and lower PGE₂ concentrations in urine than the aspirin-tolerant asthma group. During the reaction, the urinary concentrations of leukotriene E₄ and PGD₂ metabolites correlatively increased, but with markedly different patterns of the mediator release, in the aspirin-intolerant asthma group and the anaphylaxis group, respectively. The urinary PGD₂ metabolites and primary PGs were significantly decreased in the aspirin-tolerant asthma group. Urinary *ent*-PGF₂α concentrations were significantly increased in the anaphylaxis group but not the aspirin-intolerant asthma group.

Conclusions: When assessed by urinary 2,3-dinor-9α,11β-PGF₂, PGD₂ overproduction during aspirin-intolerant bronchoconstriction was clearly identified, regardless of COX inhibition. It is evident that free radical-mediated PG

generation is involved in the pathophysiology of anaphylaxis. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Aspirin, asthma, anaphylaxis, cysteinyl-leukotriene, 2,3-dinor-9α,11β-PGF₂, *ent*-PGF₂α, urine

Arachidonic acid metabolites such as cysteinyl leukotrienes (CysLTs)¹ and prostaglandins (PGs)² play important roles in the pathophysiology of various inflammatory diseases. Because these compounds are rapidly metabolized *in vivo*, the concentration of urinary metabolites provides a time-integrated estimate of the production of the parent compounds, allowing us to detect their generation *in vivo*.³ Urinary leukotriene (LT) E₄ is the most reliable analytical biomarker for monitoring the endogenous synthesis of CysLTs.⁴ Similarly, urinary 9α,11β-PGF₂ and the later appearing 2,3-dinor-9α,11β-PGF₂ are relatively major metabolites of PGD₂.^{5,6} (see this article's Fig E1 in the Online Repository at www.jacionline.org). Among the LTC₄-producing cells such as eosinophils, basophils, and mast cells, it is only the mast cell that produces significant quantities of PGD₂.⁷ Urinary PGD₂ metabolites are markedly increased in patients with mastocytosis,^{8,9} so the urinary PGD₂ metabolites are evidently useful biomarkers of mast cell activation.^{5,6} Furthermore, the measurement of PGD₂ metabolites represents a more sensitive strategy than measurement of serum tryptase.^{10,11} However, recently, some problems have arisen about whether quantification of urinary 9α,11β-PGF₂ concentrations provides a valid assessment of systemic PGD₂ production, as follows:

1. Concentrations of 9α,11β-PGF₂ were below the detection limit whereas the concentrations of 2,3-dinor-9α,11β-PGF₂ were markedly higher in human urine when estimated by liquid chromatography-mass spectrometry/mass spectrometry.¹²
2. 2,3-Dinor-9α,11β-PGF₂ showed a marked cross-reactivity (about 10%) to the antibody against 9α,11β-PGF₂ in commercially available enzyme immunoassay (EIA).⁵

These findings lead us to speculate that urinary 2,3-dinor-9α,11β-PGF₂ concentration might be a more reliable indicator in determining PGD₂ production *in vivo*. In contrast, quantification of primary PGs in urine has been shown to reflect predominantly renal production but not systemic PG production.¹³

There is another pathway that arachidonic acid is metabolized *in vivo* by a free radical-mediated mechanism to yield a series of PG-like compounds termed isoprostanes independent of the catalytic activity of the COX enzyme.¹⁴ In contrast with COX-derived PGs, which is an optically pure, free radical-mediated

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Abbreviations used

AIA:	Aspirin-intolerant asthma
ATA:	Aspirin-tolerant asthma
CysLT:	Cysteinyl leukotriene
EIA:	Enzyme immunoassay
<i>ent</i> -PGF ₂ α:	Prostaglandin F ₂ α enantiomer
GC-MS:	Gas chromatography–mass spectrometry
GC-MS-NCI:	Gas chromatography–mass spectrometry–negative-ion chemical ionization
L-ASA:	Lysine-aspirin
LT:	Leukotriene
NSAID:	Nonsteroidal anti-inflammatory drug
PG:	Prostaglandin
PGEM:	13,14-Dihydro-15keto-prostaglandin E ₂
Tetranor-PGDM:	11,15-Dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid
Tetranor-PGEM:	9,15-Dioxo-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid

peroxidation of arachidonic acid generates a racemic mixture of PGs. Thus, the presence of the enantiomer to COX-derived PG demonstrates that the PG is generated via a free radical-mediated mechanism¹⁵ (see this article's [Table E1](#) in this article's Online Repository at www.jacionline.org). The concentration of PG enantiomer in urine is a reliable index of systemic isoprostane and lipid peroxidation.^{15,16} Quantification of urinary PGF₂α enantiomer (*ent*-PGF₂α) actually provides a valuable tool to assess oxidant stress in cigarette smokers and in subjects with hypercholesterolemia.¹⁶

The clinical syndrome aspirin-intolerant asthma (AIA) is characterized by aspirin/nonsteroidal anti-inflammatory drug (NSAID) intolerance, bronchial asthma, and chronic rhinosinusitis with nasal polyposis.^{17,18} Aspirin/NSAID-induced asthma reactions are developed through the pharmacological effect of COX-1 inhibitors,^{19,20} which are accompanied by CysLTs and PGD₂ release.^{21,22} Inhaled PGE₂ protects against both aspirin-induced bronchoconstriction and the massive release of urinary LTE₄,^{23,24} so a critical deficiency in PGE₂ “braking” has been postulated as 1 possible mechanism for the AIA reaction.²⁵ The plasma aspirin concentration is adequate to inhibit COX²⁶; however, the reasons for the discrepancy between COX inhibition by aspirin and the PGD₂ overproduction in AIA are unknown. The pathophysiologic response of anaphylaxis is also produced by inflammatory mediators such as CysLTs and PGD₂ released possibly from mast cells.²⁷ Considering the finding that human mast cells generate intracellular reactive oxygen species that are functionally linked to mast cell activation,²⁸ we hypothesized that a free radical-mediated mechanism might be also responsible for PGD₂ production during acute exacerbation of anaphylaxis and AIA. There has been no comparative study of urinary arachidonic acid metabolites including the free radical-mediated PG generation comprehensively evaluated by validated methods between AIA and anaphylaxis.

In this study, a new biomarker of the urinary *ent*-PGF₂α was used to confirm the involvement of another pathway for PG production via a free radical-mediated mechanism, leading to the measurement of its counterparts PGF₂α and PGE₂ in urine as indicators of renal production. In addition, we used gas chromatography–mass spectrometry–negative-ion chemical ionization (GC-MS-NCI) for the quantification of 2,3-dinor-9α,11β-PGF₂

in urine from subjects with anaphylaxis. Song et al¹² have recently recommended the measurement of the 11,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM) concentration by liquid chromatography–mass spectrometry/mass spectrometry to be a sensitive biomarker of PGD₂ production. However, tetranor-PGDM could not be chosen for gas chromatography–mass spectrometry (GC-MS) analysis because the compound has 2 keto groups. Subsequently, we evaluated the characteristic profile of eicosanoid generation through the COX pathway and/or free radical-mediated isoprostane pathway between anaphylaxis and AIA.

METHODS

An extended version of this article's [Methods](#) section is available in the Online Repository at www.jacionline.org.

Subjects and study design

We conducted a hospital-based prospective study from January 2007 to July 2008. As shown in [Table I](#), anaphylactic subjects (n = 8) were enrolled in study I (urinary biomarkers in anaphylaxis). The diagnosis of anaphylaxis was based on the international criteria.^{29,30} We excluded subjects with vasodepressor syncope, systemic mastocytosis, hereditary angioedema, psychiatric disorders, and cardiovascular or respiratory events. None with AIA or aspirin-intolerant urticaria/angioedema type were included in study I. Adult subjects with asthma (n = 17) clinically requiring a lysine-aspirin (L-ASA) provocation test because of a suspicion of intolerance to aspirin/NSAIDs were included in study II (comparison study of AIA and aspirin-tolerant asthma [ATA] groups). The diagnosis of asthma and asthma severity were based on the American Thoracic Society criteria³¹ and the Global Initiative for Asthma guidelines,³² respectively. Subjects with an upper respiratory tract infection in the previous 6 weeks, renal or liver dysfunction, hypertension, or autoimmune disease were excluded from this study. All patients' characteristics are shown in [Table I](#). Permission to conduct the study was obtained from the Ethics Committee of the Sagami National Hospital, and all of the subjects who participated in this study gave written informed consent.

Provocation test

We performed a single-blind provocation test for the diagnosis of a specific cause of anaphylaxis^{27,29} or aspirin intolerance^{22,33} by a previously described method. Although all medications were withheld for at least 24 hours before the day of provocation to the furthest extent possible, certain medications were frequently needed to stabilize bronchial airways in the patients with severe AIA.^{34,35} As shown in [Table I](#), subjects with asthma producing a fall in FEV₁ of 20% or greater compared with the baseline were assigned as AIA (n = 10; mean cumulative dose of L-ASA, 60 mg). Subjects receiving even the maximum provocative dose of aspirin (cumulative dose of L-ASA, 375 mg) without significant bronchoconstriction or other adverse symptom were assigned as having ATA (n = 7). The subjects with anaphylaxis did not receive L-ASA or NSAID agents in the 4 weeks preceding the study or after the provocation test.

Assay of LTE₄, 9α,11β-PGF₂, PGE₂, PGF₂α, and *ent*-PGF₂α

Urine samples were collected at baseline (between 8:00 and 10:00 AM) and 0 to 3, 3 to 6, 6 to 9, and 9 to 24 hours after the provocation test.^{36,37} As shown in [Tables E1](#) and [E2](#) in this article's Online Repository at www.jacionline.org, the samples were analyzed by EIA (Cayman Chemical, Ann Arbor, Mich) for urinary concentrations of LTE₄,^{38,39} 9α,11β-PGF₂,⁴⁰ PGE₂, PGF₂α, and *ent*-PGF₂α after purification with HPLC.^{22,39} Recovery of the eicosanoids throughout the purification procedure was more than 75%. The urinary eicosanoid concentrations were expressed as picograms per milligram of creatinine.

TABLE I. Patient characteristics

Group	AIA	ATA	Anaphylaxis	P value [‡]
Female:male (n)	8:2	7:0	3:5	NS
Age (y)*	45.1 (24-64)	59.4 (24-73)	37.5 (18-58)	.07
Age at asthma onset (y)*	35.5 (18-56)	45.4 (6-69)		NS
Atopic:nonatopic (n)	5:5	4:3	8:0	NS
Peripheral eos ($\times 10^6/L$) [†]	785 (210-1950)	350 (70-1180)	160 (60-580)	.06
FEV ₁ % pred value (%) [*]	80.2 (12.7)	81.9 (14.3)		NS
Asthma severity (n)				NS
Intermittent	0	3	1	
Mild persistent	2	1	0	
Moderate persistent	4	1	0	
Severe persistent	4	2	1	
Cause of anaphylaxis			Food, n = 6 IT, n = 1 Idiopathic, n = 1	

FEV₁% pred, FEV₁ % predicted; IT, immunotherapy; Peripheral eos, peripheral eosinophil number.

*Mean (SD).

[†]Median (range).

[‡]for the comparison between AIA and ATA groups.

Quantification of 2,3-dinor-9 α ,11 β -PGF₂ by GC-MS-NCI

After purification by HPLC under the conditions shown in Table E2, 2,3-dinor-9 α ,11 β -PGF₂ was quantified with GC-MS-NCI by using ¹⁸O-labeled 2,3-dinor-9 α ,11 β -PGF₂ as the internal standard.^{41,42} (Detailed procedures are shown in the Methods section of this article's Online Repository at www.jacionline.org.)

Statistical analysis

The analysis of urinary eicosanoid concentration was performed after applying a log transformation, and the data are expressed as the medians and interquartile ranges. One-way repeated-measures ANOVA with *post hoc* analysis of the Dunn test was performed for the time-course provocation test. When the urinary data at baseline in the 3 groups (AIA, ATA, and anaphylaxis groups) were compared, the Kruskal-Wallis test followed by the Mann-Whitney test with the Bonferroni correction was carried out. In all other calculations, an unpaired *t* test or Man-Whitney test was used. The Spearman rank correlation test was used to evaluate the correlated data. *P* values of less than .05 were regarded as statistically significant.

RESULTS

An extended version of this article's Results section is available in the Online Repository at www.jacionline.org

Validation of the quantification of 2,3-dinor-9 α ,11 β -PGF₂ in urine

The detailed results can be found in this article's Figs E2 and E3 in the Online Repository at www.jacionline.org.

Study I: Urinary biomarkers in anaphylaxis

Time-course of urinary concentrations of LTE₄, 2,3-dinor-9 α ,11 β -PGF₂ and 9 α ,11 β -PGF₂. During acute exacerbation of anaphylaxis, urinary concentrations of LTE₄, 2,3-dinor-9 α ,11 β -PGF₂, and 9 α ,11 β -PGF₂ significantly increased above the baseline concentrations (baseline vs during 0-3 h: 6.5-fold for LTE₄; 20-fold for 2,3-dinor-9 α ,11 β -PGF₂; 11-fold for 9 α ,11 β -PGF₂; *P* < .05; Fig 1). The pattern of changes in the urinary 2,3-dinor-9 α ,11 β -PGF₂ concentrations was similar to that of the urinary 9 α ,11 β -PGF₂ concentrations. However, 2,3-dinor-9 α ,11 β -PGF₂ is a more predominant metabolite of PGD₂ than 9 α ,11 β -PGF₂ in urine (*P* < .01). There were significant correlations among the urinary concentrations of LTE₄, 2,3-dinor-9 α ,11 β -PGF₂, and 9 α ,11 β -PGF₂ in the anaphylaxis group (LTE₄ vs 2,3-dinor-9 α ,11 β -PGF₂, *r*_s = 0.79, *P* < .01; LTE₄ vs 9 α ,11 β -PGF₂, *r*_s = 0.44, *P* < .05; 2,3-dinor-9 α ,11 β -PGF₂ vs 9 α ,11 β -PGF₂, *r*_s = 0.68, *P* < .01; Fig 2).

Time-course of urinary concentrations of PGE₂, PGF₂ α , and ent-PGF₂ α . Primary PGD₂ is present in extremely small quantities in urine (data not shown). At baseline, the urinary ent-PGF₂ α concentrations were significantly higher than the urinary PGF₂ α concentrations (*P* < .05; Fig 1; Table II). Urinary ent-PGF₂ α concentrations 0 to 3 hours after the onset of acute exacerbation of anaphylaxis were significantly higher than baseline concentrations (2.6-fold increase; *P* < .05; Fig 1). There were no changes in the PGE₂ and PGF₂ α concentrations after the acute exacerbation of anaphylaxis.

Considering the result of study I, 2,3-dinor-9 α ,11 β -PGF₂ in urine is evidently a sensitive marker of mast cell activation related to IgE-allergic reactions. In addition, ent-PGF₂ α in urine is a useful marker of systemic oxidant inflammation *in vivo*. In study II, by using these urinary parameters, we performed a comparison of the urinary eicosanoid concentrations in the AIA and ATA groups.

Study II: Comparison study of AIA and ATA

Time-course of urinary concentrations of LTE₄, 2,3-dinor-9 α ,11 β -PGF₂, and 9 α ,11 β -PGF₂. The basal urinary LTE₄ concentrations in the AIA group were significantly higher than in the ATA group (*P* < .01; Table II). There were no significant differences in basal urinary concentrations of 2,3-dinor-9 α ,11 β -PGF₂ and 9 α ,11 β -PGF₂ between the AIA and ATA groups. As shown in Fig 3, the urinary LTE₄ concentrations after the L-ASA provocation test in the AIA group significantly increased above the basal concentrations (compared with baseline concentrations: 9.3-fold increase during 0-3 h; 8.0-fold during 3-6 h; 5.4-fold during 6-9 h; *P* < .05). No significant increase in the urinary LTE₄ concentrations in the ATA group was observed. After the provocation test, the urinary concentrations of 2,3-dinor-9 α ,11 β -PGF₂ and 9 α ,11 β -PGF₂ significantly increased above the baseline concentrations in the AIA group (baseline vs during 0-3 h: 3.8-fold increase for 2,3-dinor-9 α ,11 β -PGF₂; 3.0-fold increase for 9 α ,11 β -PGF₂; *P* < .05). In contrast, the urinary concentrations of 2,3-dinor-9 α ,11 β -PGF₂ and 9 α ,11 β -PGF₂ significantly decreased below the baseline concentrations in the ATA group (baseline vs during 3-6 h: about 80% decrease; *P* < .05). There were significant correlations among the urinary concentrations of LTE₄, 2,3-dinor-9 α ,11 β -PGF₂, and 9 α ,11 β -PGF₂ in the AIA group (LTE₄ vs 2,3-dinor-9 α ,11 β -PGF₂, *r*_s = 0.44,

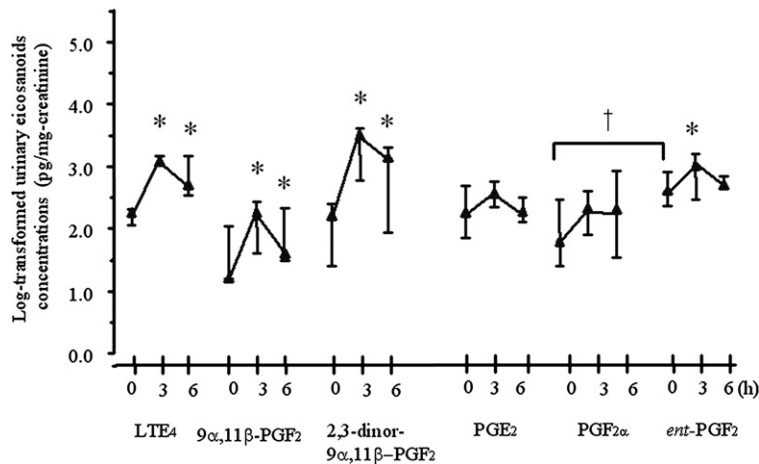


FIG 1. Time-course of urinary eicosanoid concentrations in the anaphylaxis group. The log-transformed urinary eicosanoid concentrations are expressed as medians and interquartile ranges. Anaphylaxis groups are denoted by closed triangles. * $P < .05$ compared with baseline values. † $P < .05$ for the comparison between the urinary $\text{PGF}_{2\alpha}$ and $\text{ent-PGF}_{2\alpha}$ concentrations. Urine samples were collected at baseline and after the reactions.

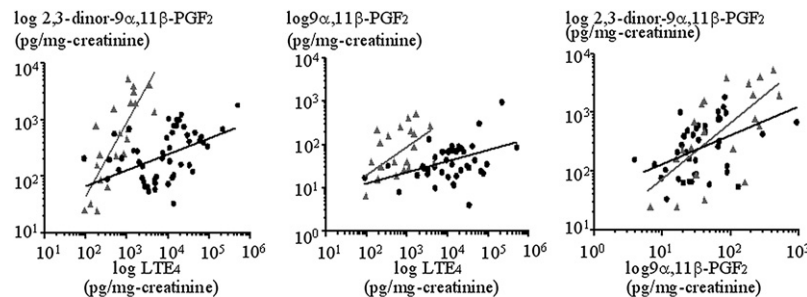


FIG 2. Significant correlations among urinary concentrations of LTE_4 , 2,3-dinor- $9\alpha,11\beta\text{-PGF}_2$ and $9\alpha,11\beta\text{-PGF}_2$ in the AIA and anaphylaxis groups. Urinary eicosanoids values are expressed by using the log scale. AIA and anaphylaxis groups are denoted by the closed circles and triangles, respectively.

TABLE II. Urinary eicosanoid concentrations at baseline in AIA, ATA, and anaphylaxis groups

Group	AIA	ATA	Anaphylaxis
LTE_4	2275 (449-3495)*,‡	156 (152-209)	183 (124-197)
2,3-Dinor- $9\alpha,11\beta\text{-PGF}_2$	161 (96.3-200)	92.5 (58.9-116)	160 (29.0-249)
$9\alpha,11\beta\text{-PGF}_2$	21.6 (18.1-31.0)	31.6 (13.7-100)	16.4 (16.0-75.4)
PGE_2	71.9 (43.0-161)†	313 (228-504)	202 (103-384)
$\text{PGF}_{2\alpha}$	89.8 (40.5-307)	87.1 (68.6-146)	71.1 (30.1-241)
$\text{ent-PGF}_{2\alpha}$	405 (356-676)	480 (359-657)	411 (290-494)

Data are expressed as medians (interquartile ranges) picograms of milligram of creatinine.

* $P < .01$ compared with baseline values in anaphylaxis group.

† $P < .05$, and ‡ $P < .01$ compared with baseline values in ATA group.

$P < .01$; LTE_4 vs $9\alpha,11\beta\text{-PGF}_2$, $r_s = 0.35$, $P < .05$; 2,3-dinor- $9\alpha,11\beta\text{-PGF}_2$ vs $9\alpha,11\beta\text{-PGF}_2$, $r_s = 0.41$, $P < .01$; Fig 2).

Time-course of urinary concentrations of PGE_2 , $\text{PGF}_{2\alpha}$, and $\text{ent-PGF}_{2\alpha}$. At baseline, the urinary PGE_2 concentration in the AIA group was significantly lower than the ATA group ($P < .05$, Fig 4; Table II). There were no changes in PGE_2 , $\text{PGF}_{2\alpha}$, or $\text{ent-PGF}_{2\alpha}$ concentrations after the L-ASA provocation test in the AIA group. The urinary PGE_2 and

$\text{PGF}_{2\alpha}$ concentrations during the periods of 0 to 3 and 3 to 6 hours after the L-ASA provocation test in the ATA group significantly decreased (baseline vs during 0-3 h or during 3-6 h: 56% and 64% decrease for PGE_2 and 70% and 71% decrease for $\text{PGF}_{2\alpha}$, respectively; $P < .05$; Fig 4). There was no decrease in urinary $\text{ent-PGF}_{2\alpha}$ concentrations by L-ASA administration in the ATA group.

Comparative study of the ratio of the urinary concentrations of LTE_4 to PGD_2 metabolites between the AIA and anaphylaxis groups

Although the urinary concentrations of LTE_4 and the PGD_2 metabolites (2,3-dinor- $9\alpha,11\beta\text{-PGF}_2$ and $9\alpha,11\beta\text{-PGF}_2$) correlatively increased during the acute exacerbation in the anaphylaxis group and the AIA group (Fig 2), there were significant differences in the ratio of the urinary concentrations of LTE_4 to 2,3-dinor- $9\alpha,11\beta\text{-PGF}_2$ or $9\alpha,11\beta\text{-PGF}_2$ between these 2 groups (Table III).

DISCUSSION

The current study has provided for the first time indirect evidence that the urinary concentration of 2,3-dinor- $9\alpha,11\beta\text{-PGF}_2$ is a useful urinary biomarker of mast cell activation. In this

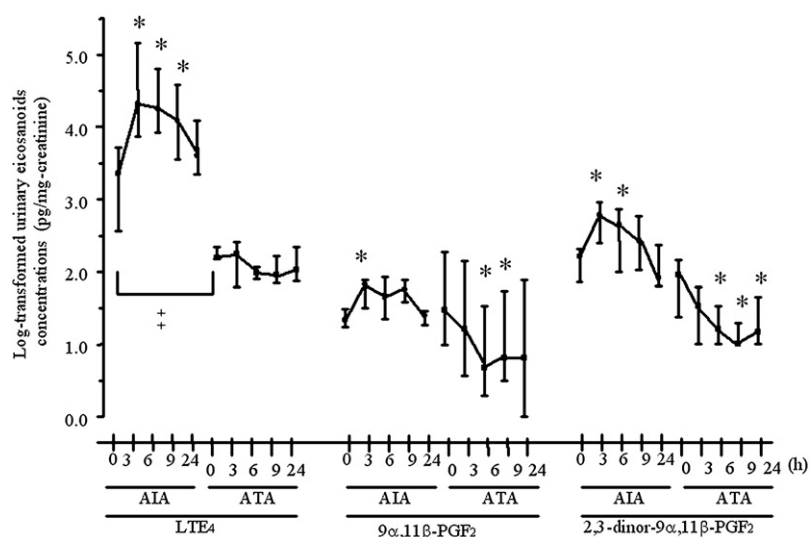


FIG 3. Time-course of urinary concentrations of LTE₄, 2,3-dinor-9α,11β-PGF₂ and 9α,11β-PGF₂ in the AIA and ATA groups. AIA and ATA are denoted by closed circles and squares, respectively. The results are expressed as in Fig 1. **P* < .05 compared with each respective baseline values. †*P* < .01 in comparison of baseline values between the AIA group and the ATA group.

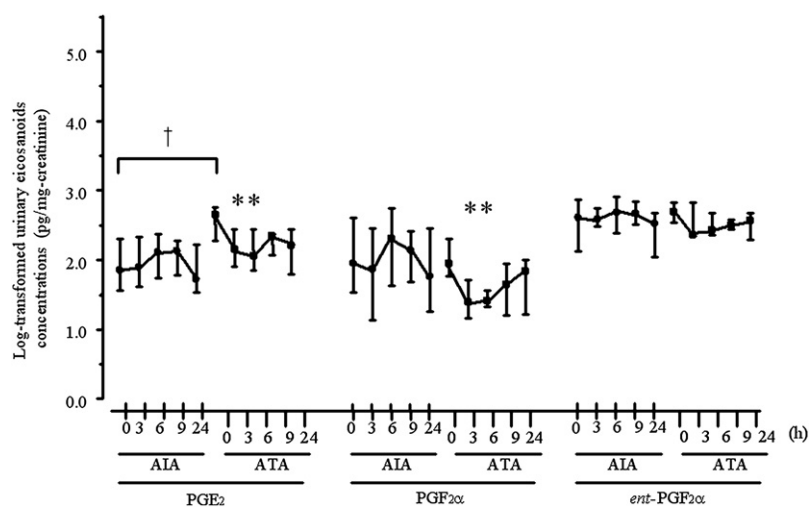


FIG 4. Time-course of urinary concentrations of PGE₂, PGF₂α and ent-PGF₂α in the AIA and ATA groups. Abbreviations and the results are expressed as in Fig 3. †*P* < .05 in the comparison of the baseline values between the AIA group and the ATA group.

way, the time-course measurements of a combination of multiple arachidonic acid metabolites in noninvasive samples of urine provide a valuable rationale for potential therapeutic targets in inflammatory diseases. As previously reported by Song et al,¹² the current study also demonstrated that the urinary 9α,11β-PGF₂ concentrations, as quantified by EIA after HPLC purification, were from 5-fold to 10-fold lower than urinary 2,3-dinor-9α,11β-PGF₂ concentrations, suggesting that 2,3-dinor-9α,11β-PGF₂ is a more predominant metabolite of PGD₂ in human urine and a more reliable marker than 9α,11β-PGF₂ in determining PGD₂ production *in vivo*. Considering that 2,3-dinor-9α,11β-PGF₂ displayed approximately 10% cross-reactivity to the antibody against 9α,11β-PGF₂ in commercially available EIA, the concentrations of 9α,11β-PGF₂ by EIA without HPLC

purification may be largely affected by 2,3-dinor-9α,11β-PGF₂ and other substances.^{6,7} It is apparent that HPLC purification is required before quantification of urinary 9α,11β-PGF₂. Bochenek et al^{10,21} have demonstrated that the time-course of 9α,11β-PGF₂ concentrations in serum is appropriate for the assessment of mast cell activation *in vivo*. However, the possibility that quantification of 9α,11β-PGF₂ might not be distinguishable from F₂-isoprostanes, even using selective ion monitoring of GC-MS, has been suggested.¹² Taken together, we conclude that (1) urinary 2,3-dinor-9α,11β-PGF₂ by GC-MS seems to be a more sensitive and appropriate biomarker of PGD₂ production than urinary 9α,11β-PGF₂, and (2) quantification of 9α,11β-PGF₂ by EIA in combination with HPLC may be more practical than the quantification by GC-MS because it is relatively laborious to analyze a

TABLE III. Ratio of urinary concentrations of LTE₄ to PGD₂ metabolites

Group Ratio	AIA			Anaphylaxis		
	Baseline	0-3 h	3-6 h	Baseline	0-3 h	3-6 h
LTE ₄ to 2,3-dinor-9 α ,11 β -PGF ₂	14.3 [†] (4.2-31.5)	66.0 [‡] (24.6-239)	98.8 ^{*,‡} (27.9-140)	3.3 (1.0-3.9)	0.9 (0.4-1.4)	2.3 (0.7-3.0)
LTE ₄ to 9 α ,11 β -PGF ₂	81.8 [†] (23.0-202)	451.2 ^{*,‡} (243-1585)	622.8 ^{*,‡} (204-1664)	11.7 (2.6-13.0)	13.6 (5.3-20.9)	7.2 (4.5-19.7)

Data are expressed as medians (interquartile ranges).

* $P < .05$ compared with each respective baseline value.

[†] $P < .05$, and [‡] $P < .01$, respectively, for the comparison of each of the respective values between the AIA group and the anaphylaxis group.

large quantity of samples with GC-MS. In the very near future, quantification of tetranor-PGDM, the most abundant PGD₂ metabolite in urine,¹² using the EIA kit that has just been made commercially available (Cayman Chemical, Ann Arbor, Mich), may be an attractive strategy.

As in previous studies,^{4,38,40,43} the AIA group exhibited significantly higher urinary LTE₄ excretion at baseline than the ATA group, even in a clinically stable condition. Baseline concentrations of LTE₄ in subjects with AIA, which may be one of the strongest predictors of AIA, are as high as the concentrations in the subjects with acute exacerbation of anaphylaxis. As opposed to the previous finding demonstrating that there is no significant difference in the major urinary PGE₂ metabolites (13,14-dihydro-15keto-PGE₂ [PGEM] and 9,15-dioxo-11 α -hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid [tetranor-PGEM]) at baseline,⁴⁴ urinary PGE₂ concentrations at baseline in the AIA group were significantly lower than the ATA group. However, the primary PGE₂ concentrations in urine have been considered to reflect predominantly local renal production.¹³ The inhibitory effect of antiasthma drugs, including inhaled corticosteroids, on PGE₂ biosynthesis may be negligible, because even a course of prednisone (60 mg/d orally) failed to inhibit the PGE₂ biosynthesis *in vivo*.⁴⁵ Interestingly, lower spontaneous production of PGE₂ has been reported in epithelial cells from nasal polyps⁴⁶ and sinonasal tissue⁴⁷ in subjects with AIA, which is associated with diminished COX-2 expression in nasal polyps⁴⁸ and the nasal mucosa.⁴⁹ Thus, it is a plausible explanation that the low COX-2 expression in kidney may result in the lower urinary PGE₂ concentration at baseline in the AIA group. On the basis of these findings, the current study supports the concept that an imbalance between the local production of PGE₂ and CysLTs at baseline is a characteristic eicosanoid profile of the pathogenesis of AIA. We also demonstrated that urinary concentrations of LTE₄ and 2 PGD₂ metabolite concentrations (2,3-dinor-9 α ,11 β -PGF₂ and 9 α ,11 β -PGF₂) were significantly increased and correlated with each other after the L-ASA provocation test in the AIA group (Fig 2). This finding provides further evidence that (1) excessive CysLTs overproduction *in vivo* during aspirin-induced bronchoconstriction is a pathophysiologic hallmark of AIA, and that (2) mast cell activation is closely associated with aspirin-induced bronchoconstriction.^{21,22,40,43} Because the anaphylaxis group did not receive L-ASA or NSAID agents in the 4 weeks preceding the study or after the provocation, the possibility should be considered that COX inhibition by the administration of L-ASA mimics an increase in PGD₂ production in only the AIA group. However, as shown in Table III, there was a significant difference in the ratio of urinary concentrations of LTE₄ to 2,3-dinor-9 α ,11 β -PGF₂ or 9 α ,11 β -PGF₂ between anaphylaxis and AIA, in spite of fact that mast cell activation is possibly involved in the pathophysiology of AIA and anaphylaxis. This finding leads us to speculate that

there may be other, unknown cellular sources besides mast cells for the CysLTs synthesis during acute reaction of AIA.

It remains unknown why mast cell-specific PGD₂ is released as a result of the administration of L-ASA to subjects with AIA in spite of the fact that the plasma L-ASA concentration was adequate for the inhibition of COX.²⁶ Recently, it was reported that a dose of aspirin ranging from 81 mg twice daily to 500 mg twice daily can induce a reduction to normal levels of urinary 9 α ,11 β -PGF₂ in subjects with systemic mastocytosis.⁹ We have examined whether PGs are generated by free radical-mediated peroxidation of arachidonic acid after the administration of L-ASA in the AIA group. The concentration of *ent*-PGF₂ α in urine is a reliable index of systemic isoprostane and lipid peroxidation.¹⁶ When PGF₂ α and *ent*-PGF₂ α were quantified by each of the respective EIA kits, which did not exhibit cross-reactivity with each other (data not shown), the concentrations of *ent*-PGF₂ α were significantly higher than PGF₂ α concentrations in human urine, because PGF₂ α is readily metabolized by 15-hydroxyprostaglandin dehydrogenase, whereas *ent*-PGF₂ α is not a substrate for this enzyme.¹⁶ There was no change in urinary *ent*-PGF₂ α concentrations after L-ASA provocation in the AIA group. It seems that PGD₂ overproduction *in vivo* in the AIA group after the L-ASA provocation test is independent of the production via the isoprostane pathway, as judged by measuring urinary *ent*-PGF₂ α concentration. In contrast, free radical-induced autoxidation is involved in the pathophysiological mechanisms of anaphylaxis. Furthermore, the finding that there was no decrease in urinary *ent*-PGF₂ α concentrations by L-ASA administration in the ATA group provides support for the idea that *ent*-PGF₂ α is not generated from COX-dependent processes. Although F₂-isoprostanes are also regarded as markers of oxidant stress *in vivo*, we suggest that *ent*-PGF₂ α may be superior to F₂-isoprostanes for the following reasons:

1. F₂-isoprostanes produced *in situ* by nonenzymatic peroxidation of arachidonic acid on phospholipids in the cell membrane⁵⁰ are slowly released after hydrolysis by phospholipase A₂ or platelet-activating acetylhydrolases.⁵¹ Thus, it is possible that the time-course of urinary F₂-isoprostane may lag behind that of PG generation via a free radical-mediated mechanism.
2. It is reported that aspirin directly stimulates an intracellular oxidative burst from human mast cells, including H₂O₂ and F₂-isoprostane generation.⁵²

The urinary concentrations of PGE₂ and PGF₂ α , as well as 2 PGD₂ metabolites (2,3-dinor-9 α ,11 β -PGF₂ and 9 α ,11 β -PGF₂), decreased to about 70% of the basal concentration after the maximum dose of L-ASA (cumulative dose of L-ASA 375 mg) in the ATA group, indicating pharmacologic effect of COX inhibition to PG synthesis by aspirin. In contrast, the markedly smaller dose of

L-ASA might account for there being no change in the urinary PGE₂ and PGF₂α concentrations after a provocation test in the AIA group. Interestingly, Mastalerz et al⁴⁴ have also reported interesting findings that the urinary concentrations of PGEM and tetranor-PGEM remain unaffected by aspirin in the AIA group, whereas oral provocation with celecoxib, a COX-2-selective inhibitor, leads to a decrease in the urinary PGEM and tetranor-PGEM concentrations in both the AIA and ATA groups. Thus, further investigations are required to determine whether the urinary PGE₂ concentration decreases after the provocation with celecoxib in the AIA group. If so, COX-1-triggered *de novo* biosynthesis of PGE₂ from inflammatory cells, such as mast cells in the kidney, is involved in the pathophysiology of AIA.⁴⁴ If the urinary PGE₂ concentration in the AIA group cannot be suppressed by celecoxib, taken together with the results after the L-ASA provocation in the current study, we can speculate that the vast majority of the PGE₂ in the AIA group is derived from the free radical-mediated peroxidation of arachidonic acid.

In conclusion, it is here shown for the first time that *ent*-PGF₂α is a biomarker of free radical-catalyzed peroxidation of arachidonic acid in urine during the acute exacerbation of anaphylaxis. When assessed by changes in the 2,3-dinor-9α,11β-PGF₂ concentration, which is a more predominant metabolite of PGD₂ than 9α,11β-PGF₂ in human urine, PGD₂ generation was more clearly identifiable during the acute exacerbation of anaphylaxis and AIA. An imbalance between PGE₂ and CysLTs biosynthesis at baseline may contribute to the severe clinical outcome of AIA. Although the current study has provided additional evidence that mast cell activation is involved in the pathophysiology of anaphylaxis²⁷ and AIA,^{21,22,40,43} there were significant differences in the ratio of the urinary concentrations of LTE₄ to 2,3-dinor-9α,11β-PGF₂ or 9α,11β-PGF₂ between these 2 groups. The precise mechanisms underlying the putative mast cell associated-PGD₂ overproduction in the AIA group, despite the administration of a COX inhibitor, remains unknown. Further investigations are required to understand better the involvement of mast cell activation and oxidant stress, respectively, in the pathophysiology of various inflammatory diseases such as aspirin/NSAIDs-induced anaphylaxis/angioedema.

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Clinical implications: A comprehensive analysis of the urinary concentration of inflammatory mediators provides a new basis for diagnostic approaches targeting the biomarkers of mast cell activation and oxidative inflammation.

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METHODS

Subjects and study design

We conducted a hospital-based prospective study from January 2007 to July 2008. Two groups of anaphylactic subjects were enrolled in study I: a group of subjects brought to our emergency department less than 2 hours after onset of the anaphylactic symptom (ED-anaphylaxis; $n = 4$) and a group of subjects with a positive history of the anaphylaxis (test-anaphylaxis; $n = 4$) that was reproduced by a provocation test as necessary. The diagnosis of anaphylaxis was based on the international criteria.^{E1,E2} We excluded subjects with vasodepressor syncope, systemic mastocytosis, hereditary angioedema, psychiatric disorders, and cardiovascular or respiratory events. None with AIA or aspirin-intolerant urticaria/angioedema type were included in study I. Adult subjects with asthma ($n = 17$) clinically requiring an L-ASA provocation test because of a suspicion of intolerance to aspirin/NSAIDs were included in study II. The diagnosis of asthma and asthma severity were based on the American Thoracic Society criteria^{E3} and the Global Initiative for Asthma guidelines,^{E4} respectively. Subjects with an upper respiratory tract infection in the previous 6 weeks, renal or liver dysfunction, hypertension, or autoimmune disease were excluded from this study. All patient characteristics are shown in Table I. Permission to conduct the study was obtained from the Ethics Committee of the Sagami National Hospital, and all of the subjects who participated in this study gave written informed consent.

Assay of LTE₄, 9 α ,11 β -PGF₂, PGE₂, PGF₂ α , and *ent*-PGF₂ α

Urine samples were collected at baseline (between 8 and 10 AM) and 0 to 3, 3 to 6, 6 to 9, and 9 to 24 hours after the provocation test.^{E5,E6} We collected first urine samples before intensive therapy from ED-anaphylaxis subjects at the emergency department within 2 hours after the onset of first anaphylactic symptoms, followed by serial urine samples during acute exacerbation and a baseline urine sample in clinical stable condition at a different visit after 7 to 14 days. As shown in Tables E1 and E2, the samples were analyzed by EIA (Cayman Chemical, Ann Arbor, Mich) for urinary concentrations of LTE₄,^{E7,E8} 9 α ,11 β -PGF₂,^{E9} PGE₂, PGF₂ α , and *ent*-PGF₂ α after purification with HPLC.^{E8,E10} Recovery of the eicosanoids throughout the purification procedure was more than 75%. The urinary eicosanoid concentrations were expressed as picograms per milligram of creatinine.

Quantification of 2,3-dinor-9 α ,11 β -PGF₂ by GC-MS-NCI

After the addition of ¹⁸O-labeled 2,3-dinor-9 α ,11 β -PGF₂ (1 ng), 2,3-dinor-9 α ,11 β -PGF₂ was extracted from urine by using an Empore C18 disk cartridge (SD type, column size 7 mm/3 mL; 3M, St Paul, Minn). After purification of the extract by HPLC under the conditions shown in Table E2, 2,3-dinor-9 α ,11 β -PGF₂ was converted to its pentafluorobenzyl ester tri-trimethylsilyl ether derivative.^{E11,E12} Briefly, the concentrations of 2,3-dinor-9 α ,11 β -PGF₂ were quantified by Shimadzu GC-MS QP2010 (Kyoto, Japan) equipped with a DB-1 capillary column (25 m, 0.25-mm internal diameter, 0.25- μ m film thickness; J&W Scientific, Folsom, Calif) in the negative ion chemical ionization mode with methane as the reagent gas. The ion source and interface temperatures were set at 250°C. The initial column temperature was maintained at 200°C for 2 minutes and increased to 250°C at 10°C/min and then to 300°C at 5°C/min. The mass spectrum of the derivative revealed 2 prominent ions. The ion at mass per electric charge of an ion (m/z) 541.32 was formed as a base ion after the elimination of the pentafluorobenzyl fragment from the molecular ion, and this ion further fragmented to the ion at m/z 451.27 after the loss of the trimethylsilyl alcohol fragment (Fig E2). The 2,3-dinor-9 α ,11 β -PGF₂ concentration was determined by measuring the fragment ions at m/z 541.32 for endogenous compounds and m/z 545.32 for the internal standard. The retention time of the derivative was approximately 13.1 minute under these conditions. The calibration curve was linear over the range of 20 pg to 3 ng. The concentration of 2,3-dinor-9 α ,11 β -PGF₂ was calculated from the calibration curve, and the concentrations were expressed as picograms per milligram of creatinine.

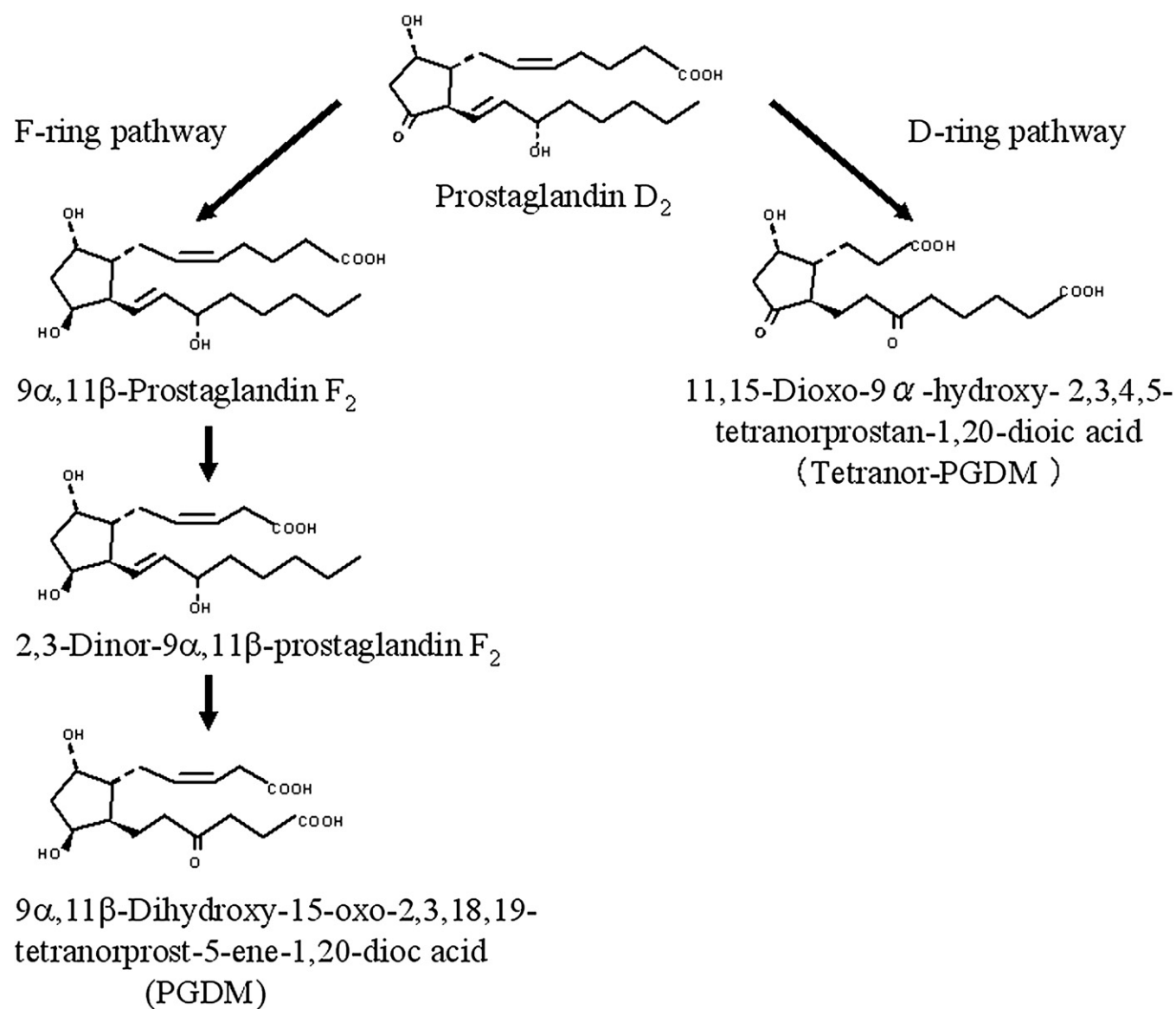
RESULTS

Validation of the quantification of 2,3-dinor-9 α ,11 β -PGF₂ in urine

The accuracy of the method was determined by adding a known amount of 2,3-dinor-9 α ,11 β -PGF₂ to 4 aliquots of the same urine specimen. The recovery rates of 2,3-dinor-9 α ,11 β -PGF₂ were 98.1% and 95.3% when urine samples were spiked with 20 and 35.5 pg of 2,3-dinor-9 α ,11 β -PGF₂, respectively. When the precision of the procedure was evaluated by analyzing samples of the same urine specimen, the coefficient of variation was within 10%. The reproducibility of the data was checked by measuring the 2,3-dinor-9 α ,11 β -PGF₂ concentrations in 2 urine samples on 2 or 3 different occasions. The concentration was 109 and 106.9 pg/mL in one urine sample and 77.8, 68.0, and 75.8 pg/mL in another sample, suggesting that the interassay variation was within 10% as calculated by the coefficient of variation. When m/z 541.32, 545.32, 451.27, and 455.27 were simultaneously quantified by selected ion monitoring in urine samples to determine the specificity of the method (Fig E2), the concentrations quantified by m/z 541.32 and 545.32 were significantly correlated with those by m/z 451.27 and 455.27 ($r^2 = 0.91$; $P < .01$; Fig E3). These findings demonstrate the specificity for urinary 2,3-dinor-9 α ,11 β -PGF₂ quantification because ions from compounds other than 2,3-dinor-9 α ,11 β -PGF₂ do not interfere.

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FIG E1. PGD₂ metabolites in urine.

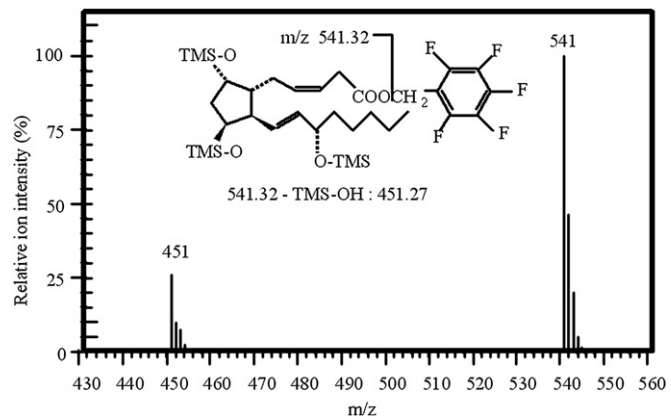


FIG E2. Mass spectrum of 2,3-dinor-9 α ,11 β -PGF₂ pentafluorobenzyl trimethylsilyl (TMS) ether derivative.

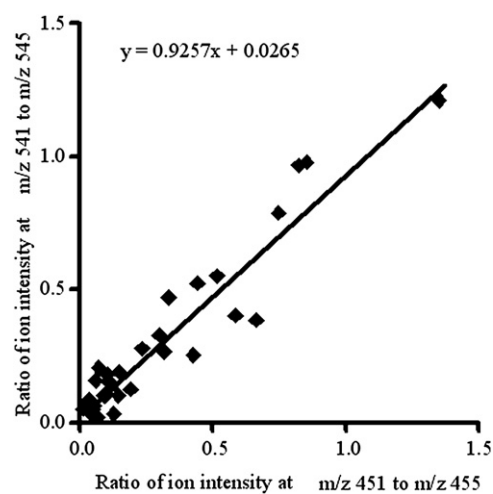


FIG E3. Specificity of determination 2,3-dinor-9 α ,11 β -PGF₂ in urine.

TABLE E1. Analytical meaning of urinary eicosanoid concentrations

Urinary eicosanoids	Metabolized compounds			Unmetabolized compounds	
	LTE ₄	9 α 11 β -PGF ₂	2,3-Dinor-9 α ,11 β -PGF ₂	Primary PGs	<i>ent</i> -PGF ₂ α
Derived from	5-LO	COX	COX	COX	Nonenzymatic
	A whole body production of			Kidney production	A free radical– mediated production
As an index of	LTC ₄	PGD ₂	PGD ₂		
Methods	HPLC/EIA	HPLC/EIA	HPLC/GC-MS	HPLC/EIA	HPLC/EIA

5-LO, 5-Lipoxygenase.

TABLE E2. HPLC conditions for separation of LTE₄ and PGs

	HPLC column HPLC mobile phase (vol/vol/vol) Peak retention time of authentic standards (min) Absorbance (nm)
2,3-Dinor-9 α ,11 β -PGF ₂	Ultrasphere C18 column (5 μ m, 4.6 mm $\times$ 15 cm) (Beckman Coulter Inc, Fullerton, Calif) Acetonitrile:distilled water:phosphoric acid: 30:70:0.1 about 5.0 210
9 α ,11 β -PGF ₂	Ultrasphere C18 column (5 μ m, 4.6 mm $\times$ 15 cm) (Beckman Coulter Inc, Fullerton, Calif) Acetonitrile:distilled water:phosphoric acid: 30:70:0.1 about 10.0 210
PGE ₂ , PGF ₂ α , and <i>ent</i> -PGF ₂ α	Ultrasphere C18 column (5 μ m, 4.6 mm $\times$ 15 cm) (Beckman Coulter Inc, Fullerton, Calif) Acetonitrile:0.05 mol/L NaH ₂ PO ₄ (pH 5.0): 30:70 PGE ₂ /PGF ₂ α and <i>ent</i> -PGF ₂ α : about 10.5/8.8/8.8 210
LTE ₄	NOVA-PACK C18 column (4 μ m, 3.9 mm $\times$ 15 cm) (Waters, Milford, Mass) Methanol: distilled water: acetic acid (65:35:0.1) containing 0.1% EDTA (pH 5.4) about 12.0 280

Flow rate of a mobile phase and column temperature: 1.0 mL/min at 35°C.