Increased urinary excretion of the prostaglandin D_2 metabolite 9α,11β-prostaglandin F_2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction

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Prostaglandin (PG)D_2 is a major product of arachidonic acid metabolism in pulmonary mast cells. We therefore attempted to determine whether measurement of the stable urinary metabolite of PGD_2, 9α,11β-PGF_2, could serve as a marker of mast cell activation in the lungs. A commercially available enzyme immunoassay was validated and found to be specific and sensitive when applied to unpurified urine. There was no diurnal variation in the levels of 9α,11β-PGF_2 in healthy volunteers. Morning baseline values of urinary 9α,11β-PGF_2 were measured in three groups—healthy volunteers (n = 9), patients with atopic asthma (n = 14), and aspirin-intolerant patients with asthma (n = 12)—and found to be very similar, 54 ± 9, 62 ± 6, and 71 ± 15 ng/mmol creatinine, respectively (means ± SEM). Urinary excretion of 9α,11β-PGF_2 was increased threefold immediately after allergen-induced bronchoconstriction in nine patients with atopic asthma. Bronchial challenge with inhaled lysine aspirin in eight aspirin-intolerant patients with asthma produced bronchoconstriction without extrapulmonary symptoms and was also followed by a significant increase in the urinary excretion of 9α,11β-PGF_2. In addition, challenge with a higher dose of aspirin produced an even greater increase in urinary 9α,11β-PGF_2, supporting dose-dependent release of PGD_2 during aspirin-induced bronchoconstriction. In contrast, the postchallenge levels of urinary 9α,11β-PGF_2 were not increased when bronchoconstriction was induced by histamine challenge in the aspirin-intolerant patients with asthma. The study confirms mast cell involvement in allergen-induced bronchoconstriction and provides novel data, which strongly support the hypothesis that pulmonary mast cells are activated during aspirin-induced airway obstruction. It is finally suggested that measurement of urinary 9α,11β-PGF_2 with enzyme immunoassay may be used as a new noninvasive strategy to monitor mast cell activation in vivo. (J Allergy Clin Immunol 1996;98:421-32.)

Key words: *Urinary 9α,11β-prostaglandin F_2, prostaglandin D_2, leukotrienes, enzyme immunoassay, mast cell, aspirin-induced asthma*

Aspirin-induced asthma (AIA) is a distinct clinical syndrome affecting a significant proportion of adult patients with asthma, in whom aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs) precipitate bronchoconstriction.[1, 2]

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Abbreviations used

AIA:	Aspirin-induced asthma
EIA:	Enzyme immunoassay
LT:	Leukotriene
NSAIDs:	Nonsteroidal antiinflammatory drugs
PG:	Prostaglandin
9α,11β-PGF_2:	9α,11β-Prostaglandin F_2, 15(S)-trihydroxy-prosta-(5Z,13E)-dien-1-oic acid
RP-HPLC:	Reverse-phase high-performance liquid chromatography

Children very rarely have AIA, whereas adults with AIA often have a particularly severe form of asthma, which is associated with rhinorrhea and recurrent polyp formation.[1, 2] In addition to bronchoconstriction and naso-ocular symptoms, ingestion of aspirin and other NSAIDs can lead to systemic hypotension, circulatory collapse, and death.[2] Although it is generally accepted that inhibition of the cyclooxygenase enzyme by aspirin or other NSAIDs provokes symptoms in sensitive patients, the precise mechanism behind the intolerance reaction remains to be established.[3]

Nevertheless, convincing evidence has recently accumulated to support a pivotal role for the leukotrienes (LTs) as mediators of aspirin-induced airway obstruction. LTs are formed from arachidonic acid through the action of the 5-lipoxygenase enzyme[4] when inflammatory cells, including eosinophils and mast cells, are activated.[5, 6] In particular, the cysteinyl LTs (LTC_4, LTD_4, and LTE_4) have been recovered from various body fluids in increased concentrations after aspirin challenge.[7-10] In addition, baseline urinary LTE_4 concentrations are significantly increased in aspirin-sensitive patients with asthma as compared with those who are tolerant of aspirin.[7, 8] The initial attempt to block aspirin-induced reactions with a cysteinyl LT-receptor antagonist showed partial inhibition in some but not all subjects.[11] However, subsequent studies with more potent receptor antagonists[12, 13] and the 5-lipoxygenase inhibitor zileuton[14] have consistently documented a substantial inhibition of NSAID-induced airway obstruction when the LT pathway is abrogated. The extrapulmonary symptoms associated with oral aspirin challenge were also attenuated after pretreatment with zileuton.[14] In addition, there is evidence to suggest that cysteinyl LTs contribute to the airway obstruction in these subjects, even when they are not exposed to NSAIDs.[15] More recently, a double-blind placebo-controlled trial has documented an improvement in pulmonary function together with a number of additional clinical variables in aspirin-intolerant patients with asthma who were treated with zileuton over a 6-week period.[16]

Despite compelling evidence that LTs are mediators of aspirin-induced bronchoconstriction and baseline airway obstruction in patients with AIA, the cellular source of the LTs has not been established. With respect to the provocation-induced release of LTs, the mast cell is one potential candidate for their formation. However, platelets, basophils, monocytes, and eosinophils have also been implicated in the pathogenesis of AIA.[2, 17] All of these cells have the capacity to generate LTs independently or through transcellular metabolism. The eosinophil count is elevated in the blood of many subjects with AIA,[1, 2, 18] and this cell is particularly competent with respect to the formation of cysteinyl LTs.[19]

In this study, we wished to evaluate the role of mast cells in the intolerance reaction to aspirin. Prostaglandin D_2 (PGD_2) is the major cyclooxygenase product released on activation of the mast cell,[20, 21] whereas few of the other implicated cells have the capacity to synthesize this prostanoid. We therefore decided to use (15S)-trihydroxy-prosta-(5Z,13E)-dien-1-oic acid (9α, 11β-PGF_2) to determine whether mast cells are activated during aspirin-induced airway obstruction. This metabolite is formed from PGD_2 in the lungs through the action of the reduced nicotinamide adenine dinucleotide phosphate–dependent enzyme 11-ketoreductase and is excreted intact in urine and constitutes one of the major urinary metabolites of PGD_2.[22]

Bronchial challenge with inhaled lysine aspirin was selected to provoke aspirin-induced bronchoconstriction because this method has been shown to be a safe and specific means to trigger a reaction that is restricted to the lungs.[23] The absence of extrapulmonary intolerance reactions was considered particularly advantageous for determining whether pulmonary mast cells are activated during aspirin-induced airway obstruction.

Urine has been found to be a useful biologic fluid with which to monitor endogenous formation of arachidonic acid metabolites, such as cysteinyl LTs and thromboxane A_2, by measuring their stable urinary metabolites.[7, 24] In addition to the easy and noninvasive access to urine, measurements in this medium are not confounded by

problems with artefactual ex vivo formation of metabolites during sampling, which could be a major problem when measuring arachidonic acid metabolites in plasma (e.g., thromboxane B_2).[25]

We have previously demonstrated that the major metabolite of the cysteinyl LTs, LTE_4, can be measured in the urine by radioimmunoassay or enzyme immunoassay (EIA), without prior purification by solid-phase extraction, followed by reverse-phase high-performance liquid chromatography (RP-HPLC).[7, 12, 26] Therefore we decided to investigate whether a similar approach could be developed for measurements of 9α,11β-PGF_2 by EIA.

In addition to the validation of the EIA for the measurement of 9α,11β-PGF_2 in urine and the application of the method to investigate the mechanisms involved in AIA, we investigated whether the early phase of allergen-induced airway obstruction in patients with atopic asthma was associated with increased urinary excretion of this metabolite. This challenge was considered a positive control because it is unambiguous that mast cell activation occurs during this reaction. Finally, to determine whether bronchoconstriction per se could trigger production of PGD_2, 9α,11β-PGF_2 was measured in the urine of some subjects before and after bronchial challenge with histamine.

METHODS

Materials

Synthetic PGD_2, $PGF_{2\alpha}$, 8-epi-$PGF_{2\alpha}$, 11-dehydrothromboxane B_2 and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ were obtained from Cayman Chemical Company (Ann Arbor, Mich.). Tritiated $PGF_{2\alpha}$ ([5,6,8,9,11,12,14,15-3H]$PGF_{2\alpha}$, 168 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). Stock solutions of histamine diphosphate for the bronchoprovocations were prepared under good laboratory practice conditions by the hospital pharmacy and kept in the refrigerator until use. Crystalline lysine aspirin (Aspisol) was purchased from Bayer AG (Leverkusen, Germany) and dissolved immediately before the challenges in saline solution (0.9% NaCl). The allergen extracts and diluents were from ALK Laboratories (Copenhagen, Denmark). Rabbit polyclonal antisera against 9α,11β-PGF_2 and LTE_4, acetylcholinesterase-linked 9α,11β-PGF_2 and LTE_4 tracer, mouse monoclonal anti-rabbit IgG, and Ellman's reagent used in the EIA were all purchased from Cayman Chemical Company. Prepacked, endcapped C_{18} silicic acid columns (Isolute) were obtained from International Sorbent Technology (Hengoed, Mid Glamorgan, U.K.). Kits from Sigma Chemical Company (St. Louis, Mo.) were used for the analysis of creatinine. EIA buffer (100 mg of sodium azide, 23.4 gm of sodium chloride, 370 mg of tetrasodium ethylenediaminetetraacetic acid, and 1 gm of bovine serum albumin per liter of 1.0 mol/L potassium phosphate buffer, pH 7.4) was prepared in the laboratory.

Bronchial provocations

A dosimeter-controlled jet nebulizer (Spira Elektro 2; Respiratory Care Center, Hämeenlinna, Finland) was used for all bronchoprovocations. The protocols have been established to have a high degree of repeatability, as described elsewhere.[12, 27] Bronchoprovocations were always performed in the mornings and started (with baseline measurements) between 7:30 and 8:30 AM. Short-acting histamine H_1-receptor antagonists were prohibited for 48 hours before a provocation, and short-acting β-agonists were not used on the morning of the challenge. Long-acting antihistamines or β-agonists (salmeterol or formoterol), disodium cromoglycate, and NSAIDs could not be used for 10 days before a challenge session. Patients being treated with inhaled steroids used their medications according to prescriptions before and after the provocations.

Pulmonary function was assessed as FEV_1 and was measured with an MDI Compact Vitalograph (Förbandsmaterial, AB, Stockholm, Sweden). After baseline measurements of FEV_1 as the best of three efforts, the bronchoprovocations were started by inhalation of 7 to 10 breaths of the diluent used for the respective provocative agent. The provocation was commenced if FEV_1 was within 10% of the baseline value after diluent and not below 50% of the predicted value. Cumulative challenges were performed by inhalation of increasing doses of the provocative agents at fixed time intervals.[12, 27] The intervals between dose increments were 3 minutes for histamine, 20 minutes for allergen, and 30 minutes for lysine aspirin. The challenges were stopped when FEV_1 had dropped by 20% or more from the baseline value obtained after inhalation of diluent. From the dose response established during the provocation, PD_{20} values (cumulated provocative doses causing a 20% fall in FEV_1) were calculated by linear interpolation between the two last cumulative doses, plotted on a 10-log scale, and defined as the intersection of this line with the point of −20% drop in FEV_1 on a linear scale.

Urine collection and subjects in the study

The total volume of urine collected at each time point was recorded, and a 50 ml aliquot was stored without addition of any preservatives at −20° C until analysis. We have previously established that the levels of LTE_4 in urine kept under these conditions remain stable when re-assayed over periods extending up to a year.[7, 26]

The allergen challenges were performed by single-dose inhalation of the PD_{20} for allergen, which had been established at a previous screening challenge. The allergen inhalation was preceded by a period of 4 to 6 hours during which pulmonary function was monitored and urine was collected at hourly intervals. The subjects (n = 9) in this part of the study were young patients with mild

atopic asthma (median age, 25 years; range, 19 to 33 years). They had a mean percent predicted FEV_1 value of 93% (range, 79% to 106%) and previously demonstrated bronchial hyperresponsiveness to histamine (PD_{20} for histamine with our method being less than 1000 μg). Their only required medication was β-agonists, administered as needed. The allergens used for the provocations were timothy grass ($n = 1$), cat dander ($n = 6$), and dog dander ($n = 2$).

The lysine aspirin provocations were part of a double-blind, placebo-controlled crossover study in which the effects of the LT-receptor antagonist MK-0679 on FEV_1 for lysine aspirin have been previously reported.[12] Urine was collected, starting about 2 hours before the challenge, and collection was continued until the subjects left the clinic (at 4:00 to 6:00 PM). Four of the eight aspirin-intolerant patients with asthma who had previously undergone the two lysine aspirin challenges were also subjected to challenge with histamine. Urine samples were collected 1 hour before the histamine challenge, and two additional samples were collected at hourly intervals after the challenge. Each subject was challenged with histamine on two occasions, separated by at least 6 weeks. In line with the clinical traits associated with asthma in aspirin-intolerant subjects, these patients were older (mean age, 45 years; range, 29 to 60 years) and had a more severe form of asthma (mean percent predicted FEV_1, 84%; range, 60% to 99%). Although the majority of subjects (75%) were being treated with relatively high doses of inhaled steroids in addition to β-mimetics, and occasionally also theophylline, all subjects had had asthma and nasal symptoms for many years.

A group of five young (21 to 24 years of age), healthy, nonasthmatic subjects supplied urine samples at 3-hour intervals during a 24-hour period. Their peak flow values were normal and showed no diurnal variation, and they had no history of asthma or atopy.

Finally, one urine sample was collected at visits to the clinic (between 7:00 and 8:00 AM) from 12 aspirin-intolerant and 14 aspirin-tolerant (atopic) patients with asthma. The baseline pulmonary function was lower in the aspirin-intolerant patients with asthma (median, 82% of predicted value; range, 58% to 99%) as compared with the aspirin-tolerant group (median, 89% of predicted value; range, 67% to 105%). They were also slightly older (median age, 45 years; range, 28 to 62 years vs median age, 38 years; range, 24 to 57 years for atopic subjects), and they generally required more asthma medication.

The study was approved by the local ethics committee, and in the case of the data from the drug trial, by the Swedish Drug Regulatory Agency (Läkemedelsverket).

EIA procedure

EIA analyses of 9α,11β-PGF_2 and LTE_4 were performed with polyclonal antisera and acetylcholinesterase-linked tracer as described previously.[28] Microtiter plates (96 wells) were coated with mouse monoclonal anti-rabbit IgG dissolved in 0.05 mol/L potassium phosphate buffer, pH 7.4 (200 μl per well). The plates were then allowed to incubate at room temperature for 18 hours, after which 100 μl of saturation buffer (200 mg sodium azide and 2 gm of bovine serum albumin per liter of EIA buffer) was added to each well. The plates were covered with plastic film, and after incubation at 4° C for an additional 18 hours, buffer was removed and the plates were ready to use, or alternatively could be stored for at least 1 month at 4° C. The 9α,11β-PGF_2/LTE_4 standards were dissolved in EIA buffer and sequentially diluted to a lowest concentration of 7.8 pg/ml. Antiserum and tracer were dissolved in 30 ml of EIA buffer (sufficient for 500 determinations). Into each well, 50 μl of standard or unpurified urine sample (in several dilutions) was pipetted in duplicate. The enzyme-linked acetylcholinesterase tracer (50 μl) was then added to all of the wells except for the total activity and blank wells. The same volume of antiserum was added to each well, excluding the total activity, nonspecific binding, and blank wells. The plates were incubated overnight in darkness at room temperature. After washing, 200 μl of Ellman's reagent (containing the enzyme substrate) was added to each of the wells. Tracer was added to the total activity wells, and absorbance was measured at 414 nm. The concentration of the sample was determined from a standard curve (standards ranged from 7.8 to 1000 pg/ml) by using the commercially available ELISA Plus software from Lab Systems (Stockholm, Sweden).

Creatinine was measured in all urine samples by a commercially available colorimetric assay by using an alkaline picrate method, and the results are expressed as nanograms of 9α,11β-PGF_2/LTE_4 per millimole of creatinine.

Validation of 9α,11β-PGF_2 immunoreactivity

Cross-reactivity studies were carried out to establish the possible displacement of the 9α,11β-PGF_2 tracer with LTE_4 (7.8 to 1000 pg/ml), 11-dehydro-thromboxane B_2 (7.8 to 16,000 pg/ml), $PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, PGD_2, and the isoprostane 8-epi-$PGF_{2\alpha}$ (7.8 to 128.000 pg/ml). Cross-reactivity was calculated from the amount of the compound required to displace 50% of the tracer bound to the antibody. Linearity was assessed in both buffer and urine by comparing measured concentrations with concentrations of synthetic 9α,11β-PGF_2 added to the samples. Precision was calculated as intra- and interassay variations, and an average value was obtained with Dahlberg's formula ($s = \sqrt{d^2/2n}$, $n >$ 80 duplicate determinations).

Purification of urine samples

Extraction of 9α,11β-PGF_2 and its separation from other urinary fatty acid metabolites was achieved by using the following protocol. Urine samples (4 ml) and buffer samples (4 ml) supplied with 1 μg of synthetic

TABLE I. Characteristics of the EIA with 9α,11β-PGF_2

Cross-reactivity (%)	
PGD_2	1.7
$PGF_{2\alpha}$	1.7
8-epi-$PGF_{2\alpha}$	<0.01
LTE_4	<0.1
13,14-dihydro-15-keto-$PGF_{2\alpha}$	<0.1
11-dehydro-thromboxane	<0.01
Detection limit (pg/ml)	8.0
Precision (%)*	9.7

*Average intra- and interassay variation calculated according to Dahlberg's formula (see Methods).

9α,11β-PGF_2 were acidified to pH 4 with 10% formic acid, and tritiated $PGF_{2\alpha}$ (10,000 dpm) was added to each sample as an internal standard. An equal volume (4 ml) of methanol was added to the samples, which were then left to precipitate at −20° C for 1 hour, followed by centrifugation (400 *g*, 5 minutes). Supernatants underwent solid-phase extraction by application on activated Isolute C_{18} columns. The columns were washed with 2 ml of water and 2 ml of 50% methanol in water, and finally 9α,11β-PGF_2 was eluted with 100% methanol (2 ml). The methanol eluates were dried under a stream of nitrogen, and residues were reconstituted in HPLC mobile phase (26/74/0.01; acetonitrile/water/acetic acid vol/vol/vol). Samples were injected into an RP-HPLC column (Nova-Pak C_{18}, 4 μm, 5100 mm Radial-Pak cartridge; Waters Chromatography, Milford, Mass.) and eluted with mobile phase at a flow rate of 1.0 ml/min. The retention time of 9α,11β-PGF_2 was determined in separate injections of an authentic standard with detection of UV absorbance of 210 nm, whereas the tritium-labeled $PGF_{2\alpha}$ was detected by liquid scintillation counting of aliquots of HPLC fractions. Determination of tritiated $PGF_{2\alpha}$ was used to assess losses of 9α,11β-PGF_2 during purification and subsequent HPLC. One-milliliter fractions, collected 15 minutes before and up to 10 minutes after the retention time of the standard 9α,11β-PGF_2, were assayed with EIA in several dilutions.

Statistics

Group mean values were compared by using Student's *t* test and were considered significantly different if the *p* value was less than 0.05. Inter- and intraassay variations were determined with Dahlberg's formula ($s = \sqrt{d^2/2n}$).

RESULTS

Characteristics of the EIA for 9α,11β-PGF_2

The specificity of the 9α,11β-PGF_2 antibody was assessed in a series of cross-reactivity studies (Table I). The antibody proved to be highly specific for 9α,11β-PGF_2, and neither $PGF_{2\alpha}$ nor 8-epi-$PGF_{2\alpha}$ caused any significant displacement of the tracer. The accuracy of the EIA for 9α,11β-PGF_2 was assessed in both buffer and urine and was excellent ($r^2 = 0.99$) (Fig. 1). The EIA was linear from 15.6 up to 200 pg/ml (Fig. 1). Samples exceeding this concentration were subsequently diluted to ensure that they fell within this region of the standard curve. The precision of the EIA according to Dahlberg's formula for intra- and interassay variations was very good (Table I).

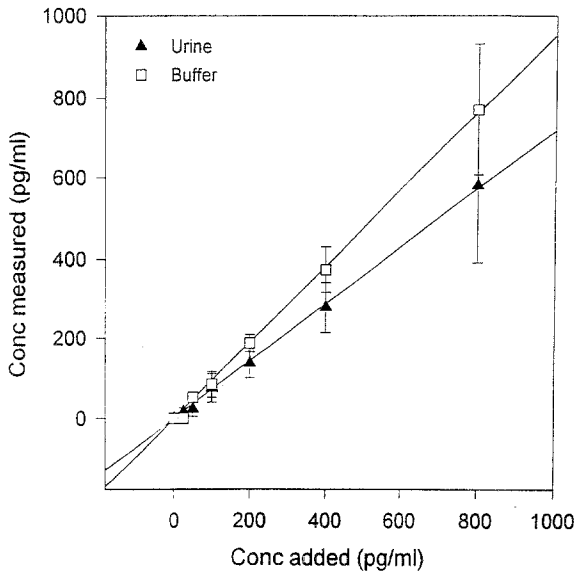


FIG. 1. Accuracy of EIA. Known amounts of synthetic 9α,11β-PGF_2 added to buffer (*open squares*) and unextracted urine (*filled triangles*) were measured with EIA. Accuracy was excellent, with $r^2 = 0.99$ in both buffer and urine (mean ± SEM, $n = 8$). *Conc,* Concentration.

Chromatographic evaluation of immunoreactivity of 9α,11β-PGF_2

Buffer supplemented with synthetic 9α,11β-PGF_2 and urine samples without any addition of standard were subjected to solid-phase extraction, followed by RP-HPLC. Immunoreactivity was assayed in HPLC fractions. In purified urine samples from patients with atopic asthma challenged with allergen, the retention time of the immunoreactivity correlated with the retention time of the synthetic standard in buffer, as shown for one of the samples (Fig. 2). Immunoreactivity was not detected in any other HPLC fractions (Fig. 2). Recovery after purification, as assessed by tritiated $PGF_{2\alpha}$, was approximately 40% in urine (42% ± 9%, $n = 8$).

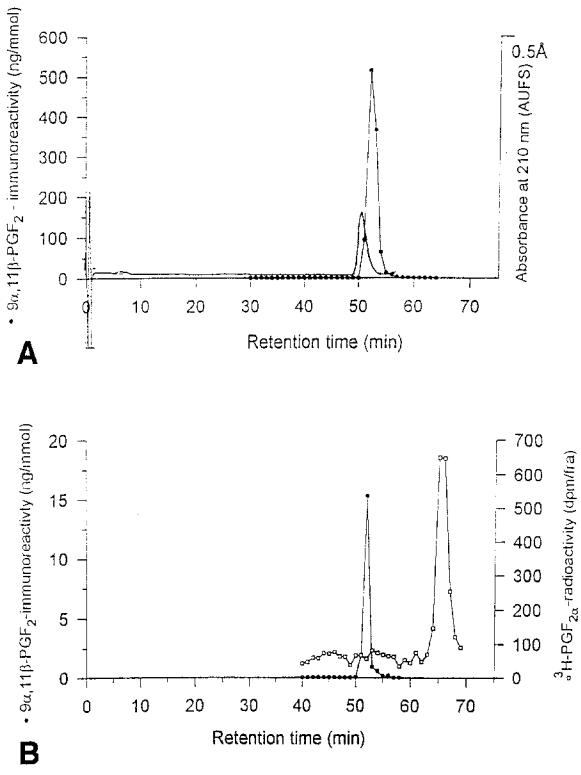


FIG. 2. Chromatographic validation of immunoreactivity. **A,** RP-HPLC of synthetic standard 9α,11β-PGF_2 in buffer, after purification by solid-phase extraction (for details see Methods). The compound was detected as EIA immunoreactivity *(filled circles)* and UV absorbance *(unbroken line).* **B,** RP-HPLC of a purified urine sample collected from a patient with atopic asthma after allergen challenge. *Filled circles* show 9α,11β-PGF_2 immunoreactivity in the fractions assessed by EIA. Losses of 9α,11β-PGF_2 during purification were corrected for by use of tritiated $PGF_{2\alpha}$ as internal standard. *Open squares* show distribution of tritiated $PGF_{2\alpha}$ as determined by liquid scintillation counting. The amount of endogenous urinary 9α,11β-PGF_2 was below the detection limit for UV absorbance measurements.

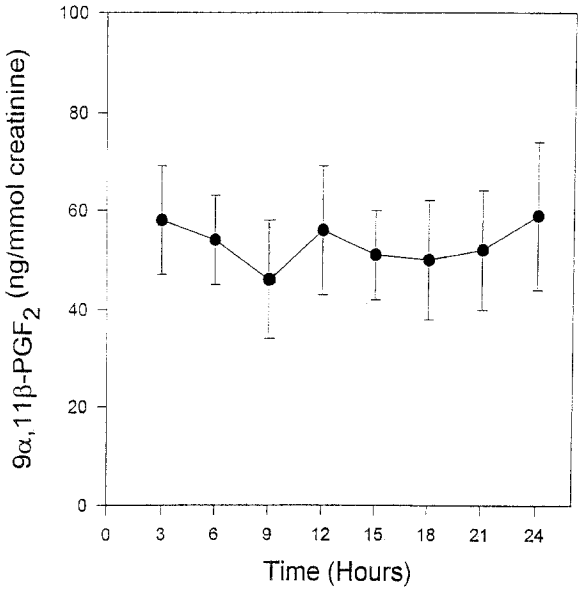


FIG. 3. EIA analysis of 9α,11β-PGF_2 *(filled circles)* in unpurified urine samples collected at three hourly intervals over a 24-hour period, from healthy nonasthmatic subjects (mean ± SEM, $n = 5$).

Basal levels of urinary 9α,11β-PGF_2 in healthy volunteers and patients with asthma

Urine samples collected from healthy nonasthmatic subjects every third hour over a 24-hour period showed no diurnal variation in the levels of 9α,11β-PGF_2 (Fig. 3). The mean morning baseline level of 9α,11β-PGF_2 was 54 ± 9 ng/mmol creatinine (mean ± SEM, $n = 5$). This was not significantly different from the baseline levels of 9α,11β-PGF_2 (at approximately 7:30 AM) determined in a group of aspirin-intolerant ($n = 12$) and a group of aspirin-tolerant ($n = 14$) patients with asthma (Table II). Previous reports on increased baseline levels of urinary LTE_4 in aspirin-intolerant patients with asthma[7, 8] were confirmed in the same samples (Table II).

Urinary 9α,11β-PGF_2 after allergen provocation

Nine patients with atopic asthma underwent bronchial challenge with a single dose of inhaled allergen. The airway response consisted of a fall in FEV_1, which began within 10 minutes after the start of the challenge and reached its peak after 30 minutes. The group mean maximum drop in FEV_1 was 21% ± 3% (mean ± SEM), and the reaction had returned to within 5% of baseline FEV_1 values after 2 hours.

Measurements of urinary 9α,11β-PGF_2 in hourly samples collected for approximately 5 hours before the allergen challenge indicated a relatively stable baseline excretion (Fig. 4). The morning baseline values (54 ± 5 ng/mmol creatinine, means ± SEM) were similar to those found in the healthy volunteers and the larger group of patients with atopic asthma (Table II). There was a nonsignificant tendency for a decrease in urinary 9α,11β-PGF_2 from the early morning value. In contrast, after the peak airway response, there was a distinct increase in the urinary concentration of 9α,11β-PGF_2 ($p < 0.05$) (Fig. 4). The mean peak increase in urinary 9α,11β-PGF_2 over baseline was 3.5-fold (immediate prechallenge value, 35 ± 5 ng/mmol creatinine vs postchallenge value, 115 ± 35 ng/

TABLE II. Basal levels of LTE_4 and 9α,11β-PGF_2 in 14 aspirin-tolerant and 12 aspirin-intolerant patients with asthma

ATA		AIA	
LTE_4	9α,11β-PGF_2	LTE_4	9α,11β-PGF_2
47 ± 6	62 ± 6	128 ± 32*	71 ± 15

Values are expressed as means ± SEM.
The data on LTE_4 in these particular subjects has previously been included as part of a published study on a larger group of subjects.
ATA, Aspirin-tolerant patients with asthma; *AIA*, aspirin-intolerant patients with asthma.
*$p < 0.001$ versus aspirin-tolerant patients with asthma.

mmol creatinine) and occurred in the sample collected immediately after the peak drop in FEV_1; the next hourly sample also contained more 9α,11β-PGF_2 than prechallenge samples (Fig. 4).

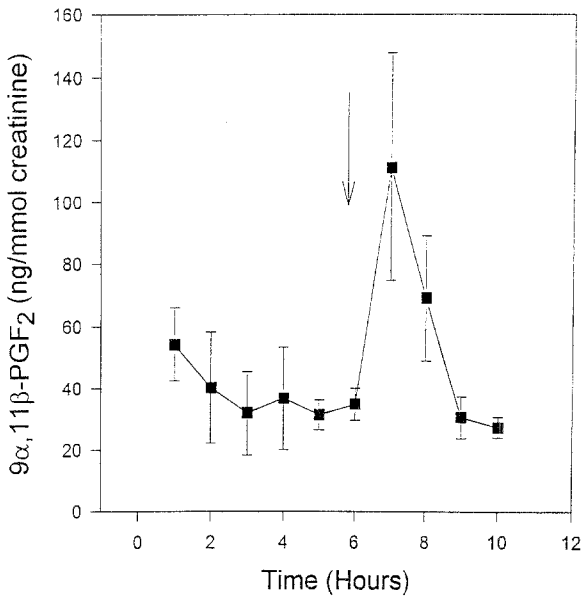


FIG. 4. Allergen-induced urinary excretion of 9α,11β-PGF_2. Mean hourly levels of 9α,11β-PGF_2 (*filled squares*) assayed in urine samples of nine patients with atopic asthma, undergoing bronchial provocation with allergen. *Arrow* indicates the time of maximum drop in FEV_1 (mean ± SEM, $n = 9$). The difference in pre- and postchallenge levels of 9α,11β-PGF_2 was statistically significant ($p < 0.05$).

Urinary 9α,11β-PGF_2 after provocation with lysine aspirin

In a placebo-controlled, double-blind crossover study, we have previously established that the oral LT-receptor antagonist MK-0679 causes a highly significant protection against the aspirin-induced bronchoconstriction in aspirin-intolerant patients with asthma.[12] The challenge involved inhalation of cumulative doses of lysine aspirin until FEV_1 had decreased by 20%, and the result of the protection was that a higher dose of lysine aspirin, expressed as a higher PD_{20} value for lysine aspirin (see Methods), could be inhaled in the presence of the LT antagonist. The influence of the two different doses of lysine aspirin on urinary 9α,11β-PGF_2 during the aspirin-induced bronchoconstriction was therefore studied (Fig. 5).

First, as in the case of the patients with atopic asthma, the baseline levels of 9α,11β-PGF_2 were in the same order (56 ± 10 ng/mmol creatinine, $n = 8$) as observed in the larger group of aspirin-intolerant patients with asthma. Second, there was a significant increase in postchallenge levels of 9α,11β-PGF_2 during both challenge sessions (Fig. 5). However, the increase in postchallenge levels of 9α,11β-PGF_2 was higher after pretreatment with MK-0679, with postchallenge levels about 2.5-fold greater than prechallenge levels (Fig. 5). The drug treatment did not affect baseline prechallenge levels of 9α,11β-PGF_2 (Fig. 5). None of the airway reactions after lysine aspirin challenge were associated with extrapulmonary symptoms.[12]

Moreover, the time course for the excretion of 9α,11β-PGF_2 during the challenge was generally very similar to that of LTE_4 (not shown). There was, however, no mathematically fixed correlation between the amounts of LTE_4 and 9α,11β-PGF_2 excreted when calculated either at the time of peak increase or for the area under the curve for postchallenge excretion of the two metabolites.

Urinary 9α,11β-PGF_2 after bronchoprovocation with histamine

Four of the aspirin-intolerant patients with asthma who previously underwent the lysine aspirin challenges were subjected to bronchial challenge with cumulative doses of histamine to determine the PD_{20}. Each subject was challenged on two different occasions, and urine was collected before and after the challenges. In contrast to the aspirin challenges, there was no postchallenge increase in urinary 9α,11β-PGF_2 after histamine challenge (Fig. 6). It is of interest that the baseline values for 9α,11β-PGF_2 in the urine samples collected before histamine and lysine aspirin challenges in the same subjects were very similar to one another (means of urinary levels of 9α,11β-PGF_2 determined on two occasions in each study for each subject: 16 vs 16.5, 37.5 vs 38.5, 114 vs 76, and 44 vs 48.5). The samples from the lysine aspirin

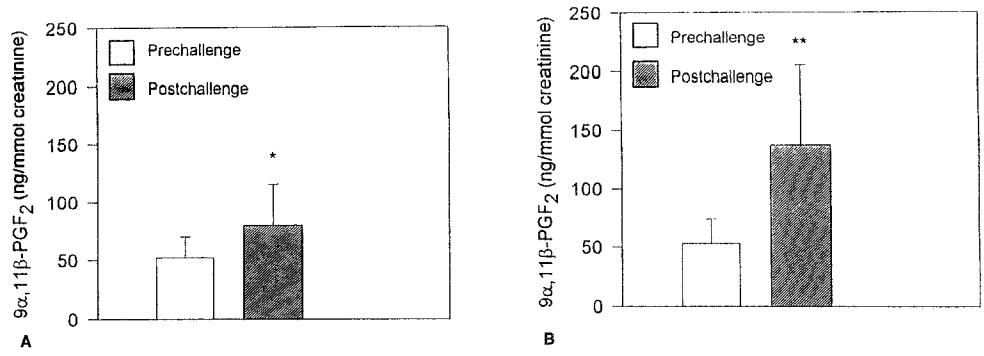


FIG. 5. Aspirin-induced urinary excretion of 9α,11β-PGF_2. Prechallenge *(open bars)* and postchallenge *(hatched bars)* levels of 9α,11β-PGF_2 in urine samples from eight patients with aspirin-intolerant asthma after challenge with lysine aspirin. **A,** Drug naive control session (placebo). **B,** After pretreatment with the LT-receptor antagonist MK-0679 (drug session). All patients received higher doses of lysine aspirin during the MK-0679 session **(B)**. The urine sample collected immediately before inhalation challenge with lysine aspirin is defined as the prechallenge level, and the peak detected after maximal airway obstruction, as the postchallenge level (mean ± SEM). Levels of statistical significance are indicated by *asterisks* (Student's *t* test, $*p < 0.05$, $**p < 0.01$).

provocations had been stored for about 2 years when analyzed for 9α,11β-PGF_2, whereas the samples from the histamine provocations were analyzed after only a few months of storage at −20° C.

DISCUSSION

It is known that PGD_2 is released in disorders involving mast cell activation, such as mastocytosis,[21] but also in association with bronchoconstriction after allergen challenge in patients with atopic asthma. The levels of PGD_2 in bronchoalveolar lavage fluid were found to be significantly increased after inhalation of[29] or endobronchial challenge with allergen.[30] Furthermore, a recent study showed increases in the levels of PGD_2, as well as its metabolite 9α,11β-PGF_2, in bronchoalveolar lavage fluid after instillation of specific allergen.[31] Basal levels of this metabolite have also been measured in the urine of healthy volunteers and after intravenous infusion of PGD_2.[22] It is of interest in the context of this investigation that two case reports on aspirin-induced shock have documented increased urinary excretion of PGD_2-M[32] and 9α,11β-PGF_2[33] and in apparent association with increased formation of other mast cell products. However, these reactions were not associated with airway obstruction. Here we present data on increased levels of 9α,11β-PGF_2 in urine after bronchoprovocation with allergen, which represents the first documentation that increased levels of this metabolite may be detected in the urine during a provocation that elicits activation of lung mast cells, and consequently, production of PGD_2. Previously, predominantly downstream PGD_2 metabolites, such as 9α-hydroxy-11,15-dioxo-tetranorprostanes, have been measured to assess urinary excretion of systemically generated PGD_2.[21]

With the exception of the case study by Butterfield et al.,[33] 9α,11β-PGF_2 has, before this study, been measured in plasma,[34] urine,[35] and bronchial lavage fluid[10, 31] by a stable isotope dilution assay with negative ion chemical ionization gas chromatography–mass spectrometry. Although this method is highly sensitive and specific, it is also time-consuming and labor-intensive, as compared with the EIA we have adopted for use on unextracted urine samples.

The specificity of the 9α,11β-PGF_2 antibody was very high with very low cross-reactivities against other arachidonic acid metabolites (Table I). Nonenzymatic oxidative formation of PGF_2 compounds termed *F_2-isoprostanes* have been shown to occur quite readily in biologic fluids.[36] The levels of these compounds in plasma rise quite dramatically with storage, even at −20° C.[37] To date, neither gas chromatography–mass spectrometry nor immunoassay has been able to distinguish between the cis cyclopentane ring hydroxyls of the F_2-isoprostanes and the trans configuration of 9α,11β-PGF_2. To determine whether nonenzymatically formed isoprostanes were contributing to the immunoreactivity measured with EIA, the cross-reactivity of the antibody with one of the most abundant F_2-isoprostanes, 8-epi-$PGF_{2\alpha}$, was tested. We could demonstrate that the cross-reactivity for 8-epi-$PGF_{2\alpha}$ in the EIA was negligible.

This may indicate that the antibody we used is unlikely to be displaced by isoprostanes in the urine to any great extent. However, we did not have the opportunity to test cross-reactivity of our antibody against other isoprostanes in this study. This issue is currently subject to further study.

The finding that the baseline values for 9α,11β-PGF_2 in samples from some of the aspirin-intolerant subjects were identical when determined in recently collected samples and samples from previous lysine aspirin challenges, however, suggests that there was no significant immunoreactivity, which could be accounted for by formation of isoprostanes or interfering autoxidation products during storage.

The specificity of the EIA was further supported by RP-HPLC analysis of urine samples, which showed that the immunoreactivity was confined to the fractions corresponding to authentic 9α,11β-PGF_2 (Fig. 2). Therefore although some interference from presently unknown products is always a possibility, the EIA was deemed sufficiently selective and suitable for use in unpurified urine samples. We have previously reported that EIA analysis of urinary LTE_4 can be performed without prepurification by HPLC.[7, 26]

Having validated the assay and documented that allergen challenge resulted in increased excretion of 9α,11β-PGF_2 into the urine, we considered the measurement of this metabolite suitable as a marker of PGD_2 formation. We therefore proceeded to investigate whether the urinary levels of 9α,11β-PGF_2 also changed during aspirin-induced bronchoconstriction.

This investigation presents data, which appear to strengthen the hypothesis that mast cells resident in the lungs are activated during aspirin-induced bronchoconstriction. First, we were able to document a clear-cut and consistent rise in urinary 9α,11β-PGF_2 after lysine aspirin provocation in all eight of the aspirin-intolerant patients with asthma studied. None of the reactions were accompanied by extrapulmonary symptoms, which lends strong support for the pulmonary formation of PGD_2. Moreover, the effect of lysine aspirin on excretion of the PGD_2 metabolite appears to be dose-dependent, because the 1.5-fold increase in urinary 9α,11β-PGF_2 after lysine aspirin challenge in a placebo situation was doubled when the same patients were challenged after pretreatment with an LT-receptor antagonist (Fig. 5). The competitive LT-receptor antagonist blocked the end organ effect of the released LTs (i.e., bronchoconstriction), and higher doses of lysine aspirin were

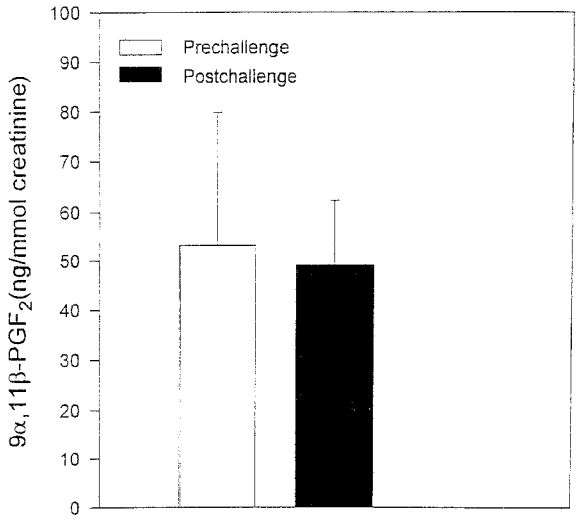


FIG. 6. No increase in urinary 9α,11β-PGF_2 after bronchoprovocation with histamine. Prechallenge *(open bar)* and postchallenge *(filled bar)* levels of 9α,11β-PGF_2 after duplicate histamine provocations in four aspirin-intolerant patients with asthma. (The result for each individual is the mean of two different provocations, done at least 6 weeks apart). Values are expressed as means ± SEM.

tolerated, which resulted in further stimulation of mediator release. We have previously reported that the group mean postchallenge increase in urinary LTE_4 was increased in these particular subjects when they were challenged in the presence of the LT-receptor antagonist.[12] In contrast, the LT-receptor antagonist did not change the basal (prechallenge) levels of 9α,11β-PGF_2 (this study) or LTE_4.[15] Considered together, the parallel and apparently dose-dependent excretion of LTE_4 and 9α,11β-PGF_2 during lysine aspirin challenge provides circumstantial evidence for the same cell being involved in the release of both compounds. The mast cell is capable of producing both PGD_2 and LTC_4,[6, 38] which makes it the only cell in the human airways known to possess the ability to synthesize both metabolites in significant quantities.

As documented by the inhibition of aspirin-induced bronchoconstriction by anti-LT drugs[11-14] and the failure of histamine-induced[7] or methacholine-induced bronchoconstriction[39] to stimulate urinary excretion of LTE_4, there is no doubt that LTs are primary mediators of aspirin-induced bronchoconstriction. Concerning excretion of 9α,11β-PGF_2, we considered the alternative explanation that PGD_2 is formed in response to the bronchoconstriction produced by the LTs, rather than during the initial intolerance reaction, which

triggers release of inflammatory mediators. There is, for example, evidence from animal models that activation of LT receptors induces formation of other cyclooxygenase products.[40, 41] However, the finding that histamine-induced bronchoconstriction did not increase urinary excretion of 9α,11β-PGF_2 strongly refutes the alternative explanation of PGD_2 being formed as a consequence of bronchoconstriction. In fact, our findings in this respect appear to be an in vivo confirmation of results obtained in the human lung in vitro in which smooth muscle constriction failed to stimulate release of PGD_2.[42] Together, the results of the measurements of 9α,11β-PGF_2 and LTE_4 during the aspirin and histamine challenges of aspirin-intolerant subjects strongly support the primary release of both LTE_4 and PGD_2 from a cellular source, which is triggered early in the intolerance reaction to aspirin. In view of the parallel release of histamine, PGD_2, LTs, and tryptase from the nasal mucosa during challenge with aspirin[9, 43, 44] and according to the results presented here, it appears most likely that mast cells in the lower airways also have a pivotal role in the intolerance reaction to aspirin.

The evidence for mast cell activation during aspirin-induced bronchoconstriction has been less than convincing. Tryptase is considered to be the most specific marker of mast cell activation.[45] Serum tryptase was found to be increased in three of seven subjects after oral aspirin administration.[43] However, the tryptase release was associated with nonrespiratory symptoms, such as flushing and gastrointestinal disturbances, indicating significant extrapulmonary activation of mast cells. Such findings are consistent with reports of patients with mastocytosis and aspirin-induced shock without bronchoconstriction.[21, 32, 33] Likewise, in an earlier study in which oral challenge with aspirin was performed,[46] three patients who experienced extrapulmonary symptoms showed elevated tryptase in serum, whereas none of the 14 subjects who had reactions limited to the lungs had detectable levels of serum tryptase. Provocations with inhaled lysine aspirin have also previously provided ambiguous data with no[47] or only a minority (three of 10) of the subjects[10] exhibiting elevated levels of tryptase in bronchoalveolar lavage fluid.

We are aware that these results introduce a conceptual paradox, namely that the cyclooxygenase inhibitor aspirin should somehow stimulate formation or release of the cyclooxygenase product PGD_2. We cannot explain this intriguing observation; nevertheless, the increased postchallenge levels of 9α,11β-PGF_2 document that aspirin-induced bronchoconstriction is associated with release of PGD_2. We are therefore left with contradictions that remain to be elucidated. On the one hand, a strong body of evidence supports cyclooxygenase inhibition as a common feature of all drugs that elicit intolerance to aspirin and other NSAIDs.[3, 17] On the other hand, these findings, as well as previous data on the release of mediators in nasal fluid,[9, 43, 44] show that the cyclooxygenase inhibitor aspirin provokes release of the cyclooxygenase product PGD_2 in aspirin-intolerant subjects. Presumably, the solution of this seemingly inconsistent set of data may provide important clues in the search for the mechanisms involved in aspirin intolerance. It should, however, be remembered that a subset of patients with systemic mastocytosis also exhibit increased formation of PGD_2, as well as histamine and tryptase, after ingestion of aspirin,[21, 33, 48] indicating that cyclooxygenase inhibition under certain circumstances may provoke mast cell activation. Finally, the similar effects of aspirin and allergen on mediator release are highly unlikely to be related to IgE involvement in the intolerance reaction to aspirin. There is no evidence of relevant IgE antibodies participating in this reaction,[49, 50] and more importantly, aspirin-intolerant patients with asthma demonstrate cross-reactivity to a great number of structurally dissimilar cyclooxygenase inhibitors.[2, 3, 17]

Concerning baseline values of urinary 9α,11β-PGF_2, urine samples collected from healthy volunteers showed no diurnal variation with respect to levels of the metabolite measured over a 24-hour period. Further, there was no difference in the basal excretion of 9α,11β-PGF_2 between the patients with asthma and the healthy volunteers or between patients with asthma who were tolerant or intolerant to aspirin. This is in contrast to the established elevations of baseline urinary LTE_4 in aspirin-intolerant patients with asthma,[7, 8, 26] which were confirmed in this study (Table II). Because it is known that eosinophils are elevated in patients with asthma and aspirin intolerance,[10, 47] it is tempting to speculate that this cell, which has an extraordinary capacity to synthesize cysteinyl LTs but not PGD_2, is the source of the increased urinary LTE_4 at baseline in aspirin-intolerant patients with asthma. The hypothesis that the mast cells are central in the intolerance reaction to aspirin and that another cell, such as the eosinophil, may explain other aspects of the pathobiology of this syndrome is in fact in line with the clinical findings that aspirin desensitization may prevent

the intolerance reaction to aspirin without corresponding major effects on the clinical course of asthma.[2]

Our observations represent the first documentation of a consistent increase in the levels of a mast cell product in subjects who experience aspirin-induced bronchoconstriction. The protocol developed to use EIA on unpurified urine should be relatively easy to automate and apply to large-scale analysis of urine, and it can be used as a new and noninvasive method to monitor in vivo mast cell activation.

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