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Prostaglandin D₂ pathway upregulation: Relation to asthma severity, control, and T_H2 inflammation

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Abstract

Background—Bronchoalveolar lavage (BAL) fluid prostaglandin D₂ (PGD₂) levels are increased in patients with severe, poorly controlled asthma in association with epithelial mast cells (MCs). PGD₂, which is generated by hematopoietic prostaglandin D synthase (HPGDS), acts on 3 G protein–coupled receptors, including chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes (CRTH2) and PGD₂ receptor 1 (DP1). However, much remains to be understood regarding the presence and activation of these pathway elements in asthmatic patients.

Objective—We sought to compare the expression and activation of PGD₂ pathway elements in bronchoscopically obtained samples from healthy control subjects and asthmatic patients across a range of disease severity and control, as well as in relation to T_H2 pathway elements.

Methods—Epithelial cells and BAL fluid were evaluated for HPGDS (quantitative real-time PCR/immunohistochemistry [IHC]) and PGD₂ (ELISA/liquid chromatography mass spectrometry) in relation to levels of MC proteases. Expression of the 2 inflammatory cell receptors DP1 and CRTH2 was evaluated on luminal cells. These PGD₂ pathway markers were then compared with asthma severity, level of control, and markers of T_H2 inflammation (blood eosinophils and fraction of exhaled nitric oxide).

Results—Confirming previous results, BAL fluid PGD₂ levels were highest in patients with severe asthma (overall $P = .0001$). Epithelial cell compartment HPGDS mRNA and IHC values differed among groups ($P = .008$ and $P < .0001$, respectively) and correlated with MC protease mRNA. CRTH2 mRNA and IHC values were highest in patients with severe asthma ($P = .001$ and $P = .0001$, respectively). Asthma exacerbations, poor asthma control, and T_H2 inflammatory markers were associated with higher PGD₂, HPGDS, and CRTH2 levels.

Conclusion—The current study identifies coordinated upregulation of the PGD₂ pathway in patients with severe, poorly controlled, T_H2-high asthma despite corticosteroid use.

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Keywords

Asthma control; chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes; prostaglandin D₂; severe asthma; T_H2 inflammation

Although severe asthma remains poorly understood,^{1–3} many studies support mast cell (MC) involvement.^{4–6} Activated MCs generate lipid mediators, particularly cysteinyl leukotrienes and prostaglandin D₂ (PGD₂),⁷ with increased PGD₂ levels recently reported in bronchoalveolar lavage (BAL) fluid from patients with severe asthma (SA), particularly in relation to frequent/severe exacerbations.⁶ PGD₂ is generated by prostaglandin D synthases after conversion of arachidonic acid to PGG₂ and PGH₂ by COXs.⁸ The lipocalin type is expressed in brain, cardiac, and adipose tissue, whereas hematopoietic prostaglandin D synthase (HPGDS) is mainly expressed in MCs, macrophages, dendritic cells, and T_H2 cells.^{8–10} Studies in patients with nasal polyps, rhinosinusitis, and eosinophilic esophagitis suggest that MCs are the predominant source of HPGDS.^{11–13}

PGD₂ acts through the 3 G protein–coupled receptors thromboxane receptor (TP), PGD₂ receptor 1 (DP1), and chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes (CRTH2/DP2), only 2 of which are found on inflammatory cells.^{9,10} Binding PGD₂ (and thromboxane) with high affinity, TP promotes smooth muscle constriction and platelet aggregation and likely contributes to allergen-induced bronchoconstriction.^{9,10} In contrast, DP1 activation causes vasodilation and bronchodilation in smooth muscle, inhibits platelet aggregation, and can promote polarization and recruitment of T_H2 lymphocytes through inhibition of T_H1 cytokines.^{14,15} CRTH2 activation on T_H2 lymphocytes, eosinophils, and basophils enhances chemotaxis and activation and potentially contributes to the development and maintenance of a T_H2 immune process.^{9,10} Additionally, CRTH2 activation of T_H2 cells induces cytokine production, which could promote IgE pathway activation on MCs, further enhancing PGD₂ generation.

On the basis of our previous findings of increased epithelial MC numbers and PGD₂ levels in SAs⁶ and recent positive CRTH2 antagonist studies in asthmatic patients,^{16–18} we hypothesized that increased PGD₂ levels would be accompanied by expression of its synthesizing enzyme, HPGDS, as well as upregulation of at least 1 of its receptors, CRTH2, on eosinophils and CD3 lymphocytes. This coordinated pathway upregulation would associate with poor asthma control, increased exacerbations, and a T_H2 immune process. To address this, epithelial and BAL cells and fluid from asthmatic patients and healthy control subjects (HCs) were evaluated for PGD₂ and HPGDS in relation to MC proteases, as well as DP1 and CRTH2 on luminal inflammatory cells. These PGD₂ pathway markers were then compared with asthma severity, level of control, and T_H2 inflammatory markers. Some results have previously been reported in abstracts.^{19–21}

RESULTS

Demographics

Bronchoscopic samples were obtained from 112 subjects, and groups did not differ by race or sex (Table I). SAs were older (overall $P < .0001$, all intergroup $P < .0002$) and had a higher body mass index compared with HCs (intergroup $P < .0001$). Less atopy and lower serum IgE levels and blood eosinophil numbers were found in HCs compared with asthmatic patients. SAs had the lowest FEV₁ percent predicted values, which were lower than in all other groups (overall $P < .0001$, all intergroup $P < .0001$). Leukotriene modifier use was more common in SAs compared with that seen in patients with milder asthma. Long-acting

β -agonist use did not differ between SAs and the Mild-Mod/ICS group. Forty (87%) SAs were using systemic corticosteroids.

All 112 subjects had BAL fluid PGD₂ measurements. Because of the availability or quality of the samples, 89 (80%) had epithelial cell brushing and 98 (87%) had BAL cell mRNA data (see Table E1 in this article's Online Repository at www.jacionline.org). Immunohistochemistry (IHC) data were available in a subset (n = 52 [epithelial] and n = 47 [BAL cell]). The subgroup with additional IHC data did not differ from those with mRNA and PGD₂ data in any basic demographic characteristic, lung function, or medication use (see Table E2 in this article's Online Repository at www.jacionline.org). In total, 88 (79%) had data from each of the 3 sample types (BAL fluid, BAL cells, and epithelial brushings; see Fig E1 and Table E3 in this article's Online Repository at www.jacionline.org for subanalysis based on the completeness of the parameters).

Severe asthma is associated with evidence of PGD₂ pathway activation

We previously reported increased PGD₂ levels in BAL fluid of SAs compared with that of HCs and patients with milder asthma, 33 of whom are included in the current analysis.⁶ The addition of 79 new subjects expanded and confirmed these prior findings because BAL fluid PGD₂ concentrations differed among the groups (overall $P = .0001$) and differentiated SAs from HCs and the Mild-Mod/ICS group (Fig 1). Analysis of the 79 nonoverlapping subjects confirmed the findings of the larger cohort and validated the findings from the previous report by Balzar et al⁶ (overall $P = .0016$, see Fig E2 in this article's Online Repository at www.jacionline.org). Because BAL fluid PGD₂ levels were generally low, LCMS confirmed PGD₂ levels in 10 subjects and correlated with ELISA-determined levels ($r_s = 0.80$, $P = .006$, see Fig E3 in this article's Online Repository at www.jacionline.org).

The PGD₂ synthesizing enzyme HPGDS is increased in the asthmatic epithelium

mRNA—Epithelial cell brushing HPGDS mRNA levels differed among the groups (overall $P = .008$), were higher in SAs compared with HCs, and tended to be higher in the Mild-Mod/ICS group compared with those seen in HCs (Fig 2, A). In all subjects epithelial cell brushing HPGDS and MC protease mRNA levels correlated ($r_s = 0.75$, $P < .001$ [tryptase]; $r_s = 0.67$, $P < .001$ [CPA3]; Fig 2, B; see Table E4 in this article's Online Repository at www.jacionline.org for MC mRNA by subject groups). In SAs (n = 35) the correlation of HPGDS with MC proteases was even stronger ($r_s = 0.88$, $P < .0001$ [tryptase]; $r_s = 0.86$, $P < .0001$ [CPA3]), suggesting a common epithelial MC source, whereas the correlation in HCs (n = 26) was less strong ($r_s = 0.48$, $P = .01$ [tryptase]; $r_s = 0.32$, $P = .11$ [CPA3]). HPGDS mRNA levels in the epithelium did not correlate with BAL fluid PGD₂ levels, even when evaluated based on severity.

IHC/protein—Epithelial brushing HPGDS staining differed across groups (total n = 52, overall $P < .0001$). The percentage of HPGDS⁺ cells was highest in SAs and differentiated SAs from both the milder asthma group and the HCs (all intergroup $P < .0004$; Fig 3, A). Staining in SAs suggested that positive cells included MCs (Fig 3, B). Epithelial cells themselves did not stain for HPGDS. HPGDS⁺ cell numbers and mRNA levels modestly correlated (n = 48, $r_s = 0.38$, $P = .008$). Similar to HPGDS mRNA levels, HPGDS⁺ cell numbers correlated with MC protease mRNA levels ($r_s = 0.40$, $P = .004$ [tryptase]; $r_s = 0.45$, $P = .001$ [CPA3]) and also correlated with BAL fluid PGD₂ levels ($r_s = 0.44$, $P = .001$). Adjustment for severity did not substantially improve these relationships.

Severe asthma is associated with increased CRTH2 receptor expression

mRNA (Fig 4)—Although DP1 mRNA levels did not differ among groups (overall $P = .88$), CRTH2 mRNA levels did differ (overall $P = .001$), being highest in SAs, significantly higher in SAs than in the Mild/no ICS group and HCs (each intergroup $P = .005$), and tending to be higher in SAs than in the Mild-Mod/ICS group (intergroup $P = .01$). Despite the lack of difference in DP1 expression, DP1 and CRTH2 mRNA levels correlated ($r_s = 0.62$, $P < .0001$ for all subjects).

IHC/protein—The percentage of DP1⁺ cells did not differ among subject groups (overall $P = .27$; Fig 5, A and B; see Table E4). Double immunostaining of BAL cells identified CRTH2⁺ cells (single positive, likely eosinophils), CD3⁺ lymphocytes, and CRTH2⁺CD3⁺ lymphocytes (double positive; Fig 5, D). The total percentage of CRTH2⁺ inflammatory cells (sum of CRTH2⁺ [single-positive] and CRTH2⁺CD3⁺ [double-positive] cells per total cells counted) was increased in SAs (overall $P = .0001$; Fig 5, C). Total CRTH2⁺ cell percentages differentiated HCs from all asthmatic groups and SAs from the Mild-Mod/ICS group (each intergroup $P = .007$). The percentages of single- and double-positive CRTH2 cells were also higher in SAs (see Table E5 in this article's Online Repository at www.jacionline.org). The percentage of CRTH2⁺ cells correlated with PGD₂ levels ($r_s = 0.46$, $P = .001$). There was no correlation between CRTH2⁺ cell numbers and mRNA levels.

Clinical implications of PGD₂ pathway markers in asthmatic patients

Asthma exacerbations—In the previous 12 months, 96% of SAs, 27% of the Mild-Mod/ICS group, and 9% of the Mild/no ICS group experienced an asthma exacerbation (overall $P < .0001$, Table I). A historical exacerbation was associated with higher BAL fluid PGD₂ levels, HPGDS⁺ cell numbers, and CRTH2⁺ cell numbers and mRNA levels compared with those seen in asthmatic patients without such a history (all $P < .05$). Epithelial HPGDS mRNA and BAL cell DP1 levels did not differ between asthmatic patients with and without exacerbations (Table II).

Poor asthma control—Eleven asthmatic patients met the NAEPP criteria² for well-controlled, 25 for not well-controlled, and 43 for very poorly controlled asthma. Although the majority of patients with very poorly controlled asthma had severe asthma, 6 patients with milder asthma were also in this category. Each control group had asthmatic patients from each severity group (see Table E6 in this article's Online Repository at www.jacionline.org). The majority of patients with very poorly controlled asthma met several criteria for poor control (70% met ≥ 2 criteria). BAL fluid PGD₂ levels, HPGDS⁺ cell numbers, and CRTH2⁺ cell numbers and mRNA levels varied by level of control, with higher values in patients with very poorly controlled asthma (all $P < .04$, Table III). PGD₂ and CRTH2 mRNA levels differentiated patients with well-controlled from those with very poorly controlled asthma (intergroup $P = .01$ and $P = .001$, respectively). HPGDS mRNA and BAL DP1 levels did not differ by level of control.

Association with T_H2 inflammation—Peripheral blood eosinophilia ($> 300/\text{mL}$) and high F_{ENO} levels (> 33 ppb, the median in these asthmatic patients), which were used as putative T_H2 inflammation markers,²⁴⁻²⁶ were evaluated in relation to PGD₂ pathway markers. Asthmatic patients with high blood eosinophil numbers had increased PGD₂ levels, HPGDS⁺ cell numbers and mRNA levels, and CRTH2 mRNA levels (all $P = .035$). Those with high F_{ENO} levels had increased PGD₂ levels ($P = .02$, see Table E7 in this article's Online Repository at www.jacionline.org). Asthmatic patients with both high F_{ENO} levels and high peripheral eosinophil numbers compared with those with low levels of both had significantly increased PGD₂ and CRTH2 mRNA levels ($P = .008$ and $P = .006$,

respectively). These data support the association of PGD₂ pathway activation with T_H2 inflammatory markers, despite the use of ICSs and even OCSs.

DISCUSSION

This study identified a coordinated upregulation of the PGD₂ pathway, from enzyme to product to receptor, particularly in patients with severe, poorly controlled, T_H2-associated asthma. Expanding on our previous study, BAL fluid PGD₂ levels, measured by means of ELISA and confirmed by using the highly sensitive and specific LCMS, were highest in SAs.⁶ Levels of the likely enzymatic source of this PGD₂, HPGDS, were also higher in the SA epithelium, correlated with its product (PGD₂), and strongly correlated with MC proteases, supporting an MC source. Although protein and mRNA levels for both PGD₂ receptors were detectable in luminal inflammatory cells from all groups, neither DP1⁺ cells nor mRNA levels differed across groups. In contrast, BAL cell CRTH2⁺ cell numbers and mRNA levels were highest in SAs and markedly different from those seen in patients with milder asthma and HCs, with increases in both single-positive (likely eosinophils and perhaps basophils) and double-positive CRTH2⁺/CD3⁺ cell numbers. Finally, upregulation of several elements of this pathway tracked with markers of T_H2 inflammation, including both F_{ENO} and blood eosinophils.

In addition to a differentiation of this pathway by using traditional severity definitions, this study was a novel evaluation of its relationship to an index of asthma control, which closely mirrors that suggested in asthma guidelines and which was able to distinguish patients with well-controlled, not well-controlled, and very poorly controlled asthma on the basis of symptoms, β -agonist use, and FEV₁. When asthmatic patients were characterized into these groups, those with the worst control again had the greatest expression and activation of this pathway, with increases in epithelial HPGDS, BAL fluid PGD₂, and luminal cell CRTH2 levels. Similarly, assessing future risk, asthmatic patients with an exacerbation in the prior 12 months had increased pathway activation compared with those without an exacerbation.

PGD₂ generation requires phospholipases to generate arachidonic acid, COXs to convert arachidonic acid to PGH₂, and prostaglandin D synthases to catalyze the isomerization of PGH₂ to PGD₂.⁸⁻¹⁰ The involvement of multiple enzymes in PGD₂ generation suggests that differences in any of these enzymes could contribute to differences in BAL fluid PGD₂ levels. However, the correlation between PGD₂ and HPGDS suggests that upregulation and activation of this enzyme plays a critical role. Unfortunately, human data on the regulation and activation of HPGDS, a glutathione-S-transferase, are limited.^{8,9} HPGDS expression and activity have been reported to be regulated by octamer-binding protein 1, although the transcription factors regulating human and murine HPGDS expression differ quite dramatically, suggesting species-specific regulation.^{9,27,28} Additionally, little is known about expressional control in MCs, and in fact, HPGDS might be constitutively expressed, suggesting a mere increase in MC numbers could increase HPGDS presence/activation.^{9,10} Data from this study strongly suggest MCs are the source of HPGDS and ultimately PGD₂ in asthmatic patients. Although the presence of MC HPGDS is well described in the nose and esophagus,¹¹⁻¹³ this is the first study linking HPGDS to the MC in asthmatic patients. Interestingly, the weaker correlations of HPGDS and MCs in the HCs suggest that PGD₂ might arise from other sources in these subjects, including macrophages.^{9,10} Finally, although a recent study reported HPGDS expression in eosinophils, very few immunostained HPGDS⁺ cells appeared to be eosinophils.²⁹

No matter the cell mix, the HPGDS product PGD₂ was confirmed to be increased in BAL fluid in patients with severe and poorly controlled asthma. However, similar to the previous report,⁶ PGD₂ levels were low, raising questions about the specificity of these ELISA-based

values. The ELISA levels were then validated in small numbers by using LCMS, correlating extremely well ($r_s = 0.80$, $P = .006$). Thus we are confident that they reflect actual values. Because PGD₂ is rapidly converted to various metabolites,^{9,10} the strong relationship of the ELISA and LCMS PGD₂ values, despite low levels, support the ongoing generation of the unstable parent compound, which is possibly indicative of chronic MC activation.¹⁰

The effects of PGD₂ depend on which of the 3 receptors is activated and its location.^{9,10} TP, which is likely responsible for PGD₂'s acute bronchoconstricting effects, is found primarily on smooth muscle and platelets and thus not studied here. DP1, which is found on a variety of cells, including smooth muscle cells, goblet cells, dendritic cells, platelets, T_H2 cells, and eosinophils, can be both anti-inflammatory and proinflammatory.^{9,10} DP1 activation causes vasodilation and bronchodilation, likely through cyclic AMP increases, whereas reports are conflicting on inflammation.^{9,10,14,15} Results of DP1 antagonist studies have been negative to date,³⁰ which is consistent with our data showing that DP1 did not differentiate asthma severity, control, exacerbation, or T_H2 inflammation.

Despite binding PGD₂ with similar affinity to DP1, CRTH2 is not structurally related and signals through a different mechanism, inhibiting adenylyl cyclase, reducing cyclic AMP, and increasing intracellular calcium levels, thereby activating chemotaxis, cell shape change, and T_H2 cytokine expression.^{9,10} In human T_H2 cells PGD₂ induced T_H2 cytokine production in a dose-dependent manner.³¹ A selective CRTH2, but not DP1, agonist mimicked PGD₂'s effects, suggesting that T_H2 activation is mediated by CRTH2 and not DP1.³¹ This is supported by our findings of positive relationships between both CRTH2 mRNA and IHC/protein with BAL fluid PGD₂ levels. Furthermore, BAL cell CRTH2 levels were strongly related to asthma severity, exacerbation, and control. Supporting these pathologic studies, a selective oral CRTH2 antagonist, OC000459, improved FEV₁ and symptoms and decreased eosinophil numbers in patients with steroid-naïve asthma.¹⁷ OC000459 also inhibited human T_H2 cell chemotaxis, cytokine production, and eosinophil activation and blunted the late asthmatic response and sputum eosinophil increase after allergen challenge.^{18,32} Recently, the CRTH2 antagonist AMG 853 did not improve symptoms or lung function in patients with moderate-to-severe asthma receiving ICSs,³³ suggesting that targeting a specific asthmatic phenotype is important for drug efficacy.

These *in vitro* and pharmacologic studies all support a strong relationship of PGD₂ and CRTH2 to T_H2 immune processes. Additionally, expression profiling studies related MCs to a T_H2 immune process in patients with steroid-naïve asthma.³⁴ To determine whether a relationship persisted in patients with more severe asthma, we compared expression of our PGD₂ pathway markers with both F_{ENO} levels and blood eosinophil numbers, which were reported to be surrogates of T_H2 inflammation.²⁴⁻²⁶ Asthmatic patients with high blood eosinophil numbers had significantly higher PGD₂, HPGDS, and CRTH2 expression than those with low eosinophil numbers. Furthermore, those with high F_{ENO} levels also had increased PGD₂ levels. These relationships persisted despite corticosteroid use. Thus our data link the PGD₂ pathway, in particular PGD₂ and CRTH2, to a T_H2 immune process, perhaps through direct effects of PGD₂/CRTH2 to activate and maintain T_H2 cells, as well as eosinophils.

This study is not without some limitations. Although SARP did not directly assess corticosteroid adherence, SAs had numerically higher levels of HPGDS, CRTH2, and PGD₂ than any other group. Thus if subjects were simply not taking their corticosteroids, these levels should have been similar to those seen in the groups with milder asthma. The asthma control proxy used here obtained symptoms, activity, medication frequency, and even FEV₁ data days if not weeks before the actual bronchoscopy, with the symptom and β -agonist scale used relying on a 3-month recall. Despite this, increases in PGD₂ pathway elements

were associated with historical worsening of asthma control and historical exacerbations, which is consistent with an association with an exacerbation-prone phenotype. Lastly, not every measurement was performed on every subject because of limited human samples, but the overall sample size for such a human-based study is still quite large.

In summary, this study presents evidence for an active PGD₂ pathway, with increases in levels of the enzyme HPGDS in association with its product, PGD₂, and one of its receptors, CRTH2, in a T_H2 asthma phenotype, specifically in association with severe poorly controlled disease. The relationship of the PGD₂-generating enzyme to the epithelial MC signature suggests that the source for this PGD₂ is the MC, whereas the increased symptoms could be due to activation, migration, or both of both eosinophils and T_H2 lymphocytes. However, much remains to be understood regarding the factors regulating and activating this pathway in asthmatic patients. Studies targeting CRTH2 antagonists in patients with severe, poorly controlled asthma with evidence for T_H2 pathway activation are needed to determine whether this pathway is critical to the maintenance or even augmentation of T_H2 inflammation and the accompanying asthma.

METHODS

Subjects, allergy testing, and spirometry

Male and female subjects 18 to 65 years of age were recruited through SARP and the Electrophilic Fatty Acid Derivatives in Asthma study. Subjects were nonsmokers in the last year and had a 5 or less pack-year smoking history. Subjects completed clinical questionnaires, allergy testing, blood draw (including complete blood count with differential and IgE measurement), and F_{ENO} measurement, as previously described.^{E1,E2} Baseline and postbronchodilator spirometry was performed according to the ATS guidelines and the SARP protocol.^{E1} Atopy was assessed by means of skin prick testing to 14 common aeroallergens. Subjects were considered atopic if there was 1 or more positive skin reaction (wheal larger than that elicited by the saline control). Serum IgE levels and complete blood counts with differentials were measured in the clinical laboratory of the University of Pittsburgh Medical Center.

Questionnaires and classification of asthma control

Information collected through questionnaires included general demographics, medical history, medication use, smoking history, and frequency of asthma symptoms and exacerbations. An asthma disease control measure was constructed to proxy for the 2007 NAEPP guidelines for those 12 years of age or older.^{E3} Five domains were analyzed by using variables captured within the clinical and medication questionnaires and baseline spirometry. Asthma control was classified into 3 groups: well controlled, not well controlled, and very poorly controlled based on daytime symptom frequency (shortness of breath, wheezing, and chest tightness), nighttime awakenings, interference with normal activity, SABA use, and FEV₁ percent predicted. Subjects rated symptoms, awakening, and SABA use frequency over the past 3 months on a 1- to 6-point scale: 1, never; 2, monthly; 3, weekly but 2 or fewer times per week; 4, more than 2 times per week but less than daily; 5, daily; and 6, 2 or more times per day. Interference with normal activity over the past 2 weeks was determined by using the Juniper AQLQ activity limitation domain.^{E4} Consistent with NAEPP guidelines, subjects were assigned to the lowest asthma control group when 1 or more of the 5 criteria for that group was met. Well-controlled asthma was classified by using the following: symptoms 2 or fewer days per week (1, 2, or 3 on frequency scale), nighttime awakenings 2 or fewer times per month (1 or 2 on frequency scale), no interference with normal activity (activity score on AQLQ of 7-5), use of SABAs 2 or fewer days per week (1, 2, or 3 on frequency scale), and FEV₁ of greater than 80% of predicted

value. Patients with not well-controlled asthma had symptoms more than 2 days per week (4 or 5 on the frequency scale), nighttime awakenings 1 to 3 times per week (3 or 4 on the frequency scale), some limitation of normal activity (activity score on AQLQ of <5-3), SABA use for symptom control on more than 2 days per week (4 or 5 on the frequency scale), and FEV₁ of 60% to 80% of predicted value. Patients with very poorly controlled asthma reported symptoms throughout the day (6 on the frequency scale), nighttime awakenings 4 or more times per week (5 or 6 on the frequency scale), extreme limitation in normal activity (activity score on AQLQ of <3-1), SABA use several times per day (6 on the frequency scale), and FEV₁ of less than 60% of predicted value.

Bronchoscopy and sample processing

During bronchoscopy, epithelial brushings from the proximal airways and BAL cells and fluid were obtained and processed according to previously published protocols and the SARP manual of procedures.^{E2,E5} BAL cells and fluid were obtained and separated by means of centrifugation at 600g.^{E1} BAL fluid from the first 100 mL was placed in aliquots and stored at -80°C for measurement of PGD₂ levels. Cells were placed in Qiazol (Qiagen) for extraction of mRNA.

BAL fluid PGD₂ measurement

ELISA—PGD₂ levels were measured by using an ELISA with commercially available components (Cayman Chemical Company) at ElisaTech, as previously described.^{E2} Samples were first purified and concentrated with C18 Sep-Pak cartridges (Waters Corp, Milford, Mass) and then derivatized per the ELISA manufacturer's instructions. The assay's limit of detection was 2 to 5 pg/mL.

LCMS—A subset of matched BAL fluid samples (n = 10) was analyzed by using LCMS to validate the PGD₂ measurements. PGD₂ was purchased from Cayman Chemical Company. Water and acetonitrile were purchased from Honeywell Burdick & Jackson (Morristown, NJ). Four milliliters of BAL fluid was used for the liquid-liquid extraction of PGD₂. Internal standard, 10 µL of 50 ng/mL [²H₉]-PGD₂, was added to each sample, followed by an equilibration time of 10 minutes. Next, 4 mL of ethyl acetate was added, and the samples were shaken for 30 minutes and then centrifuged for 10 minutes at 2900g. The organic layer was removed, dried under nitrogen, and reconstituted in 100 µL of methanol. An injection volume of 10 µL was used for analysis with LCMS.

Analyses of PGD₂ levels were conducted with a Shimadzu LC-20AD HPLC system (Columbia, Md) coupled to an AB-Sciex API-5000 triple-quadrupole mass spectrometer (Framingham, Mass). The liquid chromatography (LC) method used a Phenomenex Luna C18(2) column (2.0 mm ID × 150 mm, 3 µm) by using water with 0.1% acetic acid as mobile phase A and acetonitrile with 0.1% acetic acid as mobile phase B at a flow rate of 200 µL/min. The gradient increased from 10% B to 55% B in 45 minutes, followed by a 3-minute wash step at 100% B and back to equilibration at 10% B. The following mass spectrometer parameters were set: collision gas, 4 units; curtain gas, 40 units; ion source gas 1, 40 units; ion source gas 2, 40 units; ion spray voltage, -4.2 kV; ionization temperature, 350°C; decluster potential, -55 V; entrance potential, -5 V; collision energy, -22 eV; and collision cell exit potential, -18.4 V. Single reaction monitoring (SRM) analyses were conducted in negative electro-spray ionization (ESI) mode. The following transitions were monitored for PGD₂: m/z 351.2 (M-H⁺) → m/z 271.2 (M-H⁺-2H₂O-CO₂) and m/z 360.2 (M-H⁺) → m/z 280.2 (M-H⁺-2H₂O-CO₂) for [²H₉]-PGD₂.

PGD₂ standard was used to prepare calibration solutions at different concentrations (1, 10, 50, 100, 500, 1000, and 5000 pg of each). The corresponding [²H₉]-PGD₂ internal standard

was then added to the calibration solutions and experimental samples (500 pg). Calibration solutions underwent the same sample preparation and analytic procedures as the experimental samples, as described above. Calibration curves were calculated with a linear regression analysis of the peak area ratios of standard versus the internal standard. Prostaglandin levels were calculated by means of interpolation from the calibration curve and reported in picograms per milliliter. The assay's limit of detection for PGD₂ was 0.5 pg/mL.

qRT-PCR

Total RNA was extracted from epithelial cell brushings and BAL cells in Qiazol (Qiagen). Reverse transcription was performed with 1 µg of total RNA and random hexamers in a 50-µL reaction, according to the manufacturer's protocol (PE Applied Biosystems, Foster City, Calif). Epithelial brushing mRNA expression of HPGDS and the MC proteases tryptase and CPA3 was determined by using qRT-PCR, as previously described.^{E2} BAL cell mRNA expression for the PGD₂ receptors DP1 and CRTH2/DP2 was also evaluated by means of qRT-PCR. The primers and probes were all purchased from Applied Biosystems (Assays on Demand: HPGDS, Hs00183950_m1; tryptase, Hs02576518_gH; CPA3, Hs00157019_m1; CRTH2, Hs01867513_s1; and DP1, Hs00830594_s1). The probes were labeled with the 5'-reporter dye 6-carboxy fluorescein and the 3'-quencher dye 6-carboxy N, N, N',N'-tetramethylrhodamine. VIC-labeled ready-for-use human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe and primers were also obtained from Applied Biosystems (GenBank accession no. NM-002046; part no. 4310884E). Real-time PCR was performed on the ABI Prism 7900 sequence detection system (Applied Biosystems) in the core facilities of the University of Pittsburgh. The mRNA levels for each of the markers were determined by indexing to GAPDH with the formula $1/2\Delta Ct \times 1000$. Analysis was performed on epithelial brushing samples if the GAPDH threshold was less than 27 cycles and BAL cell samples if the GAPDH threshold was less than 30.5 cycles.

IHC

All IHC was performed on cytospin preparations fixed in 2% to 4% paraformaldehyde.

Epithelial brushing cytospin preparations for PGD₂ enzyme (HPGDS)—After the appropriate blocking steps, cytospin preparations were incubated with rabbit polyclonal antihuman HPGDS antibody (Cayman Chemical Company) at 1:100 dilution overnight at 4°C. Sections were rinsed, incubated with biotinylated secondary goat anti-rabbit antibody for 1 hour at room temperature, rinsed, and incubated in ABC reagent for 45 minutes (both from Vector Laboratories, Burlingame, Calif). Sections were developed with the chromogen 3-amino-9-ethylcarbazole (AEC) and then counterstained with hematoxylin. Slides were overlaid with Crystal Mount (Electron Microscopy Sciences, Hatfield, Pa). Slides were blinded, and numbers of HPGDS⁺ cells were counted by 2 independent observers from a total of at least 500 cells in random fields at $\times 40$ magnification with the Nikon Eclipse TS100 (Mellville, NY). The percentage of HPGDS⁺ cells was calculated by dividing the number of HPGDS⁺ cells by the total number of cells counted. Control specimens were processed in the same manner, but the primary antibody was omitted.

BAL cell cytospin preparations for PGD₂ receptors—After the appropriate blocking steps, cytospin preparations were incubated with rabbit polyclonal antibody against the DP1 receptor (Cayman Chemical Company) at 1:200 dilution overnight at 4°C. Sections were rinsed, incubated with biotinylated secondary goat anti-rabbit antibody for 1 hour at room temperature, rinsed, and incubated in ABC reagent for 45 minutes (both from Vector Laboratories). Sections were rinsed again and developed with the chromogen AEC and then counterstained with hematoxylin. Slides were overlaid with Crystal Mount (Electron

Microscopy Sciences). Slides were blinded, and the number of DP1⁺ cells was counted by 2 independent observers from a total of at least 500 cells in random fields at $\times 40$ magnification with the Nikon Eclipse TS100. The percentage of DP1⁺ cells was determined by dividing the number of DP1⁺ cells by the total number of inflammatory cells counted. Control specimens were processed in the same manner, but the primary antibody was omitted.

BAL cell cytopsin preparations were double-labeled for CRTH2 and CD3 by using sequential immunostaining with anti-CRTH2 rat mAb (1:20, BM16, Santa Cruz Biotechnology) and anti-CD3 mouse mAb (1:200, Becton Dickinson, San Jose, Calif). After blocking, cytopsin preparations were incubated with CRTH2 antibody, rinsed, incubated with biotinylated secondary rabbit anti-rat antibody followed by ABC reagent (both from Vector laboratories), and developed with AEC. After blocking with normal serum, rinsing, and treatment with the Avidin/Biotin blocking kit (Vector Laboratories), cytopsin preparations were incubated with CD3 antibody and then biotinylated secondary horse anti-mouse antibody (Vector Laboratories) for 1 hour. Slides were treated with ABC–alkaline phosphatase complex and developed with alkaline phosphatase substrate solution as the chromogen (both from Vector Laboratories). Slides were blinded, and the number of cells positive for CRTH2, CD3, or both were counted by 2 independent observers from a total of at least 600 cells, as previously described. Cells were classified as CD3⁺ only (blue staining), CRTH2⁺ only (red), CRTH2⁺/CD3⁺ double positive, or negative for either marker. Controls were performed, as previously described.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AEC	3-Amino-9-ethylcarbazole
AQLQ	Asthma Quality of Life Questionnaire
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
CPA3	Carboxypeptidase A3
CRTH2/DP2	Chemoattractant receptor–homologous molecule expressed on T _H 2 lymphocytes
DP1	PGD ₂ receptor 1
F_{ENO}	fraction of exhaled nitric oxide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HC	Healthy control subject
HPGDS	Hematopoietic prostaglandin D synthase
ICS	Inhaled corticosteroid
IHC	Immunohistochemistry

LCMS	Liquid chromatography mass spectrometry
MC	Mast cell
NAEPP	National Asthma Education and Prevention Program
OCS	Oral corticosteroid
PGD₂	Prostaglandin D ₂
qRT-PCR	Quantitative real-time PCR
SA	Patient with severe asthma
SABA	Short-acting β -agonist
SARP	Severe Asthma Research Program
TP	Thromboxane receptor

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Key messages

- Levels of several elements of the PGD₂ pathway, including the enzyme HPGDS, the CRTH2 receptor, and the end product PGD₂ are increased in patients with severe, poorly controlled asthma.
- These pathway elements associate with markers of T_H2 inflammation and exacerbations.
- The PGD₂ pathway could be a potential new therapeutic target in patients with severe, poorly controlled, T_H2-high asthma unresponsive to standard therapies.

FIG 1.
PGD₂ levels measured by using ELISA in BAL fluid samples.

FIG 2.

A, qRT-PCR of HPGDS mRNA transcripts in epithelial cell brushings relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *The difference between HCs and the Mild-Mod/ICS group tended to be different ($P = .01$) but did not reach significance. **B**, Correlation between HPGDS and tryptase epithelial cell brushing mRNA levels.

FIG 3. Epithelial brushing cell cytospin preparations. **A**, Comparison of the percentage of total HPGDS⁺ cells, as determined by means of IHC. **B**, Cytospin preparations (fixed in paraformaldehyde) stained with HPGDS antibody. *Red arrows*, HPGDS⁺ cells from an SA. Panels are shown at $\times 40$ magnification.

FIG 4.

qRT-PCR of DP1 and CRTH2 mRNA transcripts in BAL cells relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *The difference between the Mild-Mod/ICS group versus SAs tended to be different ($P = .01$) but did not reach significance.

FIG 5.

BAL cell cytospin preparations. **A** and **C**, Comparison of the percentage of total BAL cells positive for DP1 (Fig 5, A) and CRTH2 (Fig 5, C), as determined by means of IHC, is shown. **B**, Cytospin preparations stained with DP1 antibody. Red arrows, DP1⁺ cells. Upper left panel, HC; lower left panel, Mild-Mod/ICS subject; right panel, SA. Panels are shown at $\times 40$ magnification. **D**, Cytospin preparations from an SA double stained with CRTH2 and CD3 antibodies. The upper panel is shown at $\times 40$ magnification. The lower panel is shown at $\times 100$ magnification. Red arrow, CRTH2⁺/CD3⁺ lymphocyte; blue arrow, CRTH2⁺ eosinophil; black arrow, CD3⁺ lymphocyte.

TABLE I

Baseline demographic characteristics (n = 112)

	Subject group				Overall difference (P value)
	HCs (n = 33)	Mild/no ICS group (n = 11)	Mild-Mod/ICS group (n = 22)	Severe asthma (n = 46)	
Age (y)*	27 (24-39)	26 (20-31)	30 (23-39)	47 (37-54)	<.0001 ^{†//¶}
Sex, male/female	16/17	2/9	5/17	14/32	.12
Race, AA/white/other	23/3/7	6/3/2	14/5/3	37/6/3	.29
BMI (kg/m ²)*	24 (22-29)	27 (24-31)	26 (24-28)	31 (26-35)	.0007 [†]
Atopy (%)	58	100	91	76	.007 ^{‡§}
Serum IgE (kU/L)*	28 (14-65)	98 (55-252)	109 (42-405)	166 (44-501)	<.0001 ^{†‡§}
Blood eosinophils (/mL)*	100 (100-200)	200 (100-300)	200 (100-300)	300 (100-400)	.012 ^{‡‡}
Baseline FEV ₁ (% predicted)*	96 (93-105)	89 (86-97)	90 (71-105)	56 (41-71)	<.0001 ^{†//¶}
Exhaled NO (ppb)*	20 (13-34)	37 (25-66)	25 (17-35)	39 (23-81)	.0003 ^{†¶}
Hx exacerbation (%)	NA	9	27	96	<.0001 ^{//¶}
Use of LTM (no.)	NA	0	4	32	<.0001 ^{//¶}
Use of LABAs (no.)	NA	NA	15	38	NA

If not indicated otherwise, analyses were done with Pearson χ^2 tests.

AA, African American; BMI, body mass index; LABA, long-acting β -agonist; LTM, leukotriene modifier; NA, not applicable.

* Data were analyzed by using Kruskal-Wallis tests.

[†] Significant intergroup comparisons were noted as follows: HCs versus SAs

[‡] HCs versus Mild-Mod/ICS group

[§] HCs versus Mild/no ICS group

^{//} Mild/no ICS group versus SAs

[¶] Mild-Mod/ICS group versus SAs.

TABLE IIHistory of recent exacerbation and PGD₂ pathway markers in all asthmatic patients

PGD ₂ pathway marker	Recent asthma exacerbation:				<i>p</i>
	No		Yes		
	No.	Median (IQR)	No.	Median (IQR)	
BAL fluid PGD ₂ (pg/mL)	28	6.0 (4.6-8.8)	51	8.5 (6.0-19)	.001
Epithelial HPGDS mRNA	24	5.0 (1.2-8.7)	39	3.3 (1.8-9.5)	.99
HPGDS ⁺ cells in epithelium (%)	21	1.0 (0.6-2.3)	19	4.0 (2.7-6.0)	.0002
BAL cell CRTH2 mRNA	26	0.1 (0.04-0.2)	43	0.2 (0.1-1.7)	.015
CRTH2 ⁺ BAL cells (%)	19	3.9 (2.1-6.3)	16	5.9 (4.4-9.0)	.048
BAL cell DP1 mRNA	26	2.1 (1.1-6.3)	43	1.4 (0.5-9.1)	.60
DP1 ⁺ BAL cells (%)	19	4.4 (3.3-5.9)	16	4.0 (3.0-4.5)	.21

IQR, Interquartile range.

TABLE III

Level of NAEPP asthma control and PGD₂ pathway markers in all asthmatic patients

PGD ₂ pathway marker	Level of asthma control						Overall <i>P</i> value
	Well controlled		Not well controlled		Very poorly controlled		
	No.	Median (IQR)	No.	Median (IQR)	No.	Median (IQR)	
BAL fluid PGD ₂ (pg/mL)	11	6.5 (4.1-7.0)	25	7.0 (5.0- 10.5)	43	9.0 (6.0-19.0)	.014*
Epithelial HPGDS mRNA	10	6.2 (0.9-17.6)	21	4.4 (2.5-8.0)	32	3.0 (1.7-8.1)	.84
HPGDS ⁺ cells in epithelium (%)	7	1.4 (0.6-2.2)	16	2.0 (0.1-3.1)	17	3.8 (1.8-4.8)	.038
BAL cell CRTH2 mRNA	10	0.03 (0.008-0.1)	25	0.15 (0.06-0.8)	34	0.2 (0.1-1.8)	.004*
CRTH2 ⁺ BAL cells (%)	6	4.7 (3.0-5.9)	14	2.5 (1.6-6.5)	15	5.9 (4.3-9.5)	.041
BAL cell DP1 mRNA	10	0.7 (0.2-2.2)	25	3.6 (1.0-5.7)	34	1.8 (0.7-9.2)	.11
DP1 ⁺ BAL cells (%)	6	3.6 (2.7-5.4)	14	4.6 (3.3-5.9)	15	3.9 (3.3-4.6)	.35

IQR, Interquartile range.

* Significant intergroup comparisons were found between patients with well-controlled asthma and very those with poorly controlled asthma. The asthma control proxy was based on 2007 NAEPP guidelines.