

Eosinophil production of prostaglandin D₂ in patients with aspirin-exacerbated respiratory disease

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Background: Aspirin-exacerbated respiratory disease (AERD) differs from aspirin-tolerant disease in part because of eosinophilic tissue infiltration and overexpression of arachidonic acid metabolic pathway components that lead to enhanced secretion of cysteinyl leukotrienes and prostaglandin (PG) D₂ observed constitutively and paradoxically in response to aspirin and other COX inhibitors. We have previously demonstrated the capacity of IFN- γ to drive cysteinyl leukotriene expression and response.

Objective: We investigated eosinophils as a source of PGD₂ production in patients with AERD.

Methods: Eosinophils were enriched from tissue and peripheral blood obtained from control subjects, patients with aspirin-tolerant disease, and patients with AERD. mRNA was extracted and evaluated for expression of hematopoietic prostaglandin D synthase (hPGDS). Expression of hPGDS protein was confirmed with Western hybridization and immunofluorescence staining. Cells were stimulated with aspirin, and secretion of PGD₂ was quantified. CD34⁺ progenitor cells were isolated and matured into eosinophils in the presence or absence of IFN- γ and hPGDS mRNA, and PGD₂ release was measured.

Results: Gene expression analysis revealed that eosinophils from tissue and blood of patients with AERD display increased levels of hPGDS compared with asthmatic and control samples.

Western hybridization confirmed the increase in hPGDS mRNA translated to increased protein expression. Immunofluorescence confirmed mast cells and eosinophils from tissue of patients with

AERD and asthma demonstrated hPGDS expression, with higher levels in eosinophils from patients with AERD. Incubation of eosinophils from blood and tissue with aspirin stimulated PGD₂ release. IFN- γ -matured eosinophil progenitors showed enhanced hPGDS expression and increased levels of PGD₂ release at baseline and after aspirin stimulation. **Conclusions:** In addition to mast cells, eosinophils represent an important source of PGD₂ in patients with AERD and identify a new target for therapeutic intervention. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Aspirin-exacerbated respiratory disease, aspirin-tolerant asthma, chronic sinusitis, cytokines, eosinophils, prostaglandin D₂, nasal polyps

Aspirin-exacerbated respiratory disease (AERD) is a disease of the upper (chronic rhinosinusitis [CRS]/nasal polyps [NPs]) and (usually) lower (asthma) airways that differs from aspirin-tolerant asthma/chronic sinusitis by the unique sensitivity to aspirin and other nonselective COX-1 inhibitors. AERD comprises as many as 7% of patients with adult-onset asthma and up to 12% to 14% of adults with severe asthma.^{1,2} During aspirin reactions, many mediators are released, including cysteinyl leukotrienes (CysLTs), tryptase, eosinophil cationic protein (ECP), and prostaglandin (PG) D₂, suggesting both mast cell and eosinophil activation.³⁻⁵

In patients with AERD, baseline levels of PGD₂ and its metabolites are higher in the blood than in subjects who are aspirin tolerant, and after ingestion of aspirin, these levels further increase in patients with AERD.^{5,6} Aspirin desensitization followed by continual high-dose aspirin therapy is a treatment option for AERD,⁷ and if the subjects tolerate the desensitization, they benefit from improved disease control. However, not all patients with AERD can be desensitized, and those in whom desensitization fails are distinguished by their constitutive overproduction and aspirin-stimulated release of PGD₂, which correlates with the severity of airflow obstruction.⁶ In addition to defining a more severe subgroup in whom aspirin desensitization fails, PGD₂ likely contributes to the severity through its myriad of biological activities that include inducing vasodilation and vascular leakage, bronchoconstriction, and recruitment and activation of basophils, eosinophils, dendritic cells, and both T_H2-like lymphocytes and type 2 innate lymphoid cells.⁸⁻¹¹

Although both eosinophils and mast cells are critical to AERD pathogenesis, an important role for eosinophils is supported by observations made by our group^{12,13} and others¹⁴⁻¹⁶ that tissue from patients with AERD expresses approximately 3-fold greater numbers of eosinophils and ECP compared with aspirin-tolerant subjects. Historically, PGD₂ has been thought of as a mast cell

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Abbreviations used

AERD:	Aspirin-exacerbated respiratory disease
CHES:	Chronic hyperplastic eosinophilic sinusitis
CRS:	Chronic rhinosinusitis
CT:	Cycle threshold
CysLT:	Cysteinyl leukotriene
ECP:	Eosinophil cationic protein
EF1 α :	Elongation factor 1 α
hpf:	High-power field
hPGDS:	Hematopoietic prostaglandin D ₂ synthase
LT:	Leukotriene
LysASA:	Lysine aspirin
NP:	Nasal polyp
PG:	Prostaglandin
qPCR:	Quantitative real-time PCR
Siglec-8:	Sialic acid-binding immunoglobulin-like lectin 8

product; however, eosinophils can express hematopoietic prostaglandin D₂ synthase (hPGDS), the enzyme responsible for PGD₂ synthesis, and eosinophils do secrete PGD₂.¹⁷ Given that eosinophil expression greatly exceeds that of mast cells in patients with AERD, it is reasonable to speculate that eosinophils might also be an important source of PGD₂. Expression of hPGDS has been observed in NPs of patients with CRS,¹⁸ and one report suggested that a subpopulation of eosinophils in the NPs were one source of the enzyme.¹⁹

Therefore the current studies were performed to assess the production of PGD₂ by eosinophils in patients with AERD. In particular, we investigated increased expression of hPGDS in NPs of patients with AERD and whether this increase was associated with eosinophils. Recently, aspirin was shown by our research group to directly activate eosinophils and potentiate mediator release.²⁰ Therefore we investigated PGD₂ release when eosinophils from patients with AERD were stimulated with aspirin. In addition, we demonstrated that IFN- γ uniquely contributes to the development and differentiation of eosinophils, enhancing their ability to produce and release mediators, including CysLTs and eosinophil-derived neurotoxin.²¹ Therefore we also queried whether a similar result would be observed with expression of hPGDS and subsequent PGD₂ release from eosinophil progenitors matured in the presence of this cytokine.

METHODS**Subjects**

NP tissue was obtained from subjects referred to the University of Virginia Health System for sinus surgery under a protocol approved by the University of Virginia Institutional Review Board. Control tissue was harvested from the sinus cavities of patients undergoing surgery that required access to their paranasal sinuses for reasons other than chronic sinusitis (eg, orbital decompression, cerebrospinal fluid leak repair, or transphenoidal pituitary surgery). Depending on the quantity of tissue available, specimens were divided and used for subsets of the various experimental procedures outlined below. Eosinophilic sinusitis (chronic hyperplastic eosinophilic sinusitis [CHES]) was histologically defined as previously described based on the presence of 5 or more eosinophils/400 $\times$ high-power field (hpf).¹ AERD was defined by a compelling history involving a hypersensitivity reaction within 2 to 3 hours of ingestion of either aspirin or other nonsteroidal anti-inflammatory drugs. NPs were obtained from 26 subjects, including 17 with CHES and 9 with AERD, and additionally, control tissue was obtained from 9 subjects. For studies involving blood eosinophils, 10 patients with AERD, 12 asthmatic

patients, and 15 nonasthmatic control subjects were enrolled, although not all subjects were involved with every experiment (see individual figures for exact numbers). Characteristics for subjects involved in the blood draw are shown in Table E1 in this article's Online Repository at www.jacionline.org.

Immunofluorescence and antitryptase staining

Polyp tissue was fixed in 4% paraformaldehyde, paraffin embedded, and sectioned by the Histology Core Laboratory of the University of Virginia. Samples were deparaffinized and hydrated to distilled water. For immunofluorescence, heat-induced antigen retrieval was performed by heating sections for 20 minutes in citrate buffer (Abcam, Cambridge, Mass). Slides were washed and blocked with 1% BSA, 10% goat serum (Sigma, St Louis, Mo), and Fc Block (1 μ g; BD Pharmingen, Sparks, Md) for 2 hours. Specific staining for hPGDS was performed with a 1:150 dilution of a rabbit anti-human hPGDS antibody (Abcam ab89709) for 16 hours at 4°C. Sections were rinsed and then incubated with secondary allophycocyanin goat anti-rabbit IgG (1:200; Life Technologies, Grand Island, NY) for 1 hour at room temperature. Nuclei were stained with 100 ng/mL 4',6-diamidino-2-phenylindole (Sigma) for 30 minutes at room temperature. Samples were washed in PBS and aqueous mounted with VectaMount AQ (Vector Laboratories, Burlington, Calif). Then samples were analyzed by using a Zeiss AxioImager Z2 equipped with Apotome for optical sectioning (Zeiss, Thornwood, NY), and hPGDS⁺ cells were scored in a blind fashion. For antitryptase staining, rehydrated slides were treated with 3% peroxidase for 5 minutes, followed by antigen retrieval (Dako target retrieval solution, pH 9; Dako, Glostrup, Denmark). Slides were incubated overnight at 4°C with a 1:500 dilution of anti-human mast cell tryptase clone AA1 (Dako). Development of the slides was performed with the Envision+System-HRP (AEC) system (Dako), with labeled polymer added for 30 minutes and chromogen for 5 minutes, followed by counterstaining with Gill hematoxylin (Sigma) for 3 minutes. Tryptase-positive cells were scored in a blind fashion, taking the average of 10 fields per slide.

Eosinophil isolation

Eosinophils were enriched from peripheral blood by means of Ficoll-Hypaque (Sigma) density centrifugation, followed by dextran sedimentation and hypotonic lysis. Eosinophils were enriched from granulocytes by using negative magnetic affinity column purification (CD16⁻; Miltenyi Biotec, San Diego, Calif) and were greater than 95% pure, as measured by using flow cytometry.²²

Western hybridization of cellular extracts

Eosinophils were lysed. Equal amounts of protein were added to 5X Laemmli Sample Buffer (GeneScript, Piscataway, NJ) and heated for 5 minutes at 95°C. Samples were electrophoresed on 12% TruPAGE Precast Gels (Sigma) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, Mass) for 18 hours at 40V. The membrane was blocked with TBST buffer (50 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, and 0.05% Tween 20) and 5% nonfat dry milk before addition of anti-hPGDS antibodies (Abcam). The target protein was visualized with the addition of goat anti-rabbit horseradish peroxidase secondary antibody (Bio-Rad Laboratories, San Jose, Calif), after which blots were developed with the Immobilon Chemiluminescent kit (Millipore). After primary analysis, blots were stripped with 100 mmol/L glycine and reprobed with an antibody to β -actin. Analysis of blots was performed by using densitometry (GS-800 Calibrated Densitometer, Bio-Rad Laboratories) by using the ratio of hPGDS to β -actin calculated with ImageQuant TL v2005 (Amersham Biosciences, Pittsburgh, Pa).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on NP tissue, peripheral blood eosinophils, and newly differentiated eosinophils for hPGDS. For the NP studies, polyps were minced and digested with Accutase

(Innovative Cell Technologies, San Diego, Calif) for 1 hour at 37°C, and the leukocyte-containing fraction was collected by passing the cell suspension through a 70- μ m nylon mesh strainer (BD Falcon, Bedford, Mass). Eosinophils were enriched by using magnetic affinity column purification with positive selection for CD15 followed by negative selection for CD16 (Miltenyi Biotec, San Diego, Calif) and were greater than 90% pure and viable, as measured by using flow cytometry. Total RNA was extracted with TRI Reagent (Sigma). Conversion of mRNA to cDNA was performed with a TaqMan Reverse Transcription kit (Roche, Branchburg, NJ). Total RNA (200 ng) was added to each reaction along with oligo dT primers, 5.5 mmol/L MgCl₂, 2 mmol/L dNTPs, RNasin, and reverse transcriptase. Reactions occurred over 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C in a Bio-Rad iCycler thermocycler (Bio-Rad Laboratories). The PCR mix consisted of SensiMix (Bioline, Taunton, Mass), cDNA, and 200 μ mol/L of each primer. Data were analyzed as the change in cycle threshold (CT) of each cytokine transcript in comparison with either elongation factor 1 α (EF1 α) or β -actin (as appropriate based on concordance of their CT with that of the gene of interest). EF1 α primers were purchased (SABiosciences, Frederick, Md). The primers for hPGDS were as follows: sense, 5'-GGGCA GAGAAAACAAGATGT-3'; antisense, 5'-CCCCCTAAATATGTGTC CAAG-3' (Integrated DNA Technologies, Coralville, Iowa).

Eosinophil progenitor activation

PBMCs were isolated by means of Ficoll-Hypaque density centrifugation from blood obtained from 10 healthy volunteers. CD34⁺ cells were purified (>90%) from PBMCs by using positive magnetic affinity column purification (CD34⁺; Miltenyi Biotec), and eosinophil progenitors were derived by using the technique of Hudson et al.²³ culturing purified CD34⁺ cells in complete medium (RPMI 1640 and 10% FBS) supplemented with stem cell factor (25 ng/mL; BD Biosciences, San Jose, Calif), thymopoietin (25 ng/mL; R&D Systems, Minneapolis, Minn), Fms-like tyrosine kinase 3 ligand (25 ng/mL; BD Biosciences), IL-3 (25 ng/mL; BD Biosciences), and IL-5 (25 ng/mL; BD Biosciences) with or without IFN- γ (20 ng/mL; BD Bioscience) for 3 days and then culturing for an additional 3 weeks with only IL-3 and IL-5 (again with or without IFN- γ). Cells were washed, and fresh media and cytokines were applied weekly. As previously described,²¹ these cells are phenotypically and functionally similar to purified blood eosinophils. When examined by using flow cytometry, the cells were 3.1% dual positive for CCR3/sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8) without IFN- γ , and 17.7% were dual positive for CCR3/Siglec-8 with IFN- γ . The cells were examined for expression of Fc ϵ RI and CD203c as markers of basophils, and these markers were found on less than 3% of the cells. Morphologically, the newly differentiated eosinophils had bilobed nuclei and granule staining.²¹ qPCR was performed, as described above, to measure the expression of hPGDS in the differentiated eosinophils.

Functional behavior of purified blood or newly differentiated eosinophils

Purified blood or eosinophils differentiated with or without IFN- γ were either left in a resting state or stimulated with lysine aspirin (LysASA; Sanofi-Aventis, Athens, Greece; 1 and 10 mmol/L) for 30 minutes. For studies involving pretreatment with COX-1 inhibitors, eosinophils were incubated for 30 minutes with either 10 mmol/L ibuprofen sodium salt (Santa Cruz Biotechnology, Dallas, Tex) or 0.1 mmol/L SC-560 (Santa Cruz Biotechnology), after which cells were washed in PBS and stimulated with varying doses of LysASA for an additional 30 minutes. Supernatants were collected and assayed with an enzyme-linked immunoassay for PGD₂ (Cayman, Ann Arbor, Mich), according to the manufacturer's directions (lower limit of detection, 55 pg/mL).

Statistical analyses

Data were contrasted between unstimulated and stimulated cells by using the Mann-Whitney paired *t* test for nonparametric data. Control, CHES, and AERD cohorts were compared by using nonparametric unpaired *t* tests.

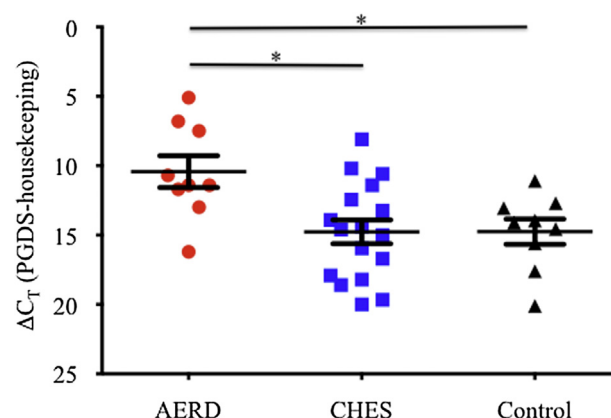


FIG 1. hPGDS gene expression in sinus tissue determined by using qPCR. Tissue samples were homogenized after surgical removal, and RNA was isolated. Transcript levels of hPGDS were quantified by using PCR with SYBR Green detection. Data (mean $\pm$ SEM) reflect relative expression of each gene in comparison with the housekeeping gene EF1 α (Δ CT). Control samples (n = 9) are depicted as black triangles, CHES samples (n = 17) as blue squares, and AERD samples (n = 9) as red circles. **P* < .02 compared with CHES and control samples.

Statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif).

RESULTS

Differential expression of hPGDS in sinus tissue

NP tissue from patients with AERD (n = 9) and patients with CHES (n = 17) along with control sinus tissue (n = 9) was digested, and RNA was extracted. Expression of hPGDS was measured by means of qPCR (Fig 1), and Δ CT for AERD was 4.34 and 4.33 cycles earlier than the housekeeping gene EF1 α for the CHES and control groups, respectively, reflecting that hPGDS transcript expression was approximately 20-fold higher (*P* < .02) in the AERD group.

Immunofluorescence staining of hPGDS in NPs

To confirm the posttranslational expression of hPGDS and to define the cellular source, we performed immunofluorescence staining on tissue samples from tissue from patients with AERD, patients with CHES, and control subjects. Representative images are shown in Fig 2, and the isotype control is displayed in Fig E1 in this article's Online Repository at www.jacionline.org. Essentially no hPGDS staining was observed in control tissue, largely reflecting the absence of inflammatory cells (Fig 2, A). Moderate hPGDS levels were detected in CHES samples (Fig 2, B), with the highest levels found in tissue from patients with AERD (Fig 2, C). As shown in the insert in Fig 2, D, most of these cells were identified as eosinophils by being granulocytes with bilobed nuclei (Fig 2, D, white arrows) or alternatively were mononuclear cells without visualizable granules, most likely mast cells (Fig 2, D, yellow arrows). The average number of hPGDS-expressing cells per hpf was determined (see Fig E2 in this article's Online Repository at www.jacionline.org). The highest numbers were found in patients with AERD, with the majority of cells identified as eosinophils, which is consistent with our previous studies.¹² Mast cell numbers in tissue sections were counted to confirm that the majority of cells observed as staining positive for hPGDS were eosinophils. Staining for mast

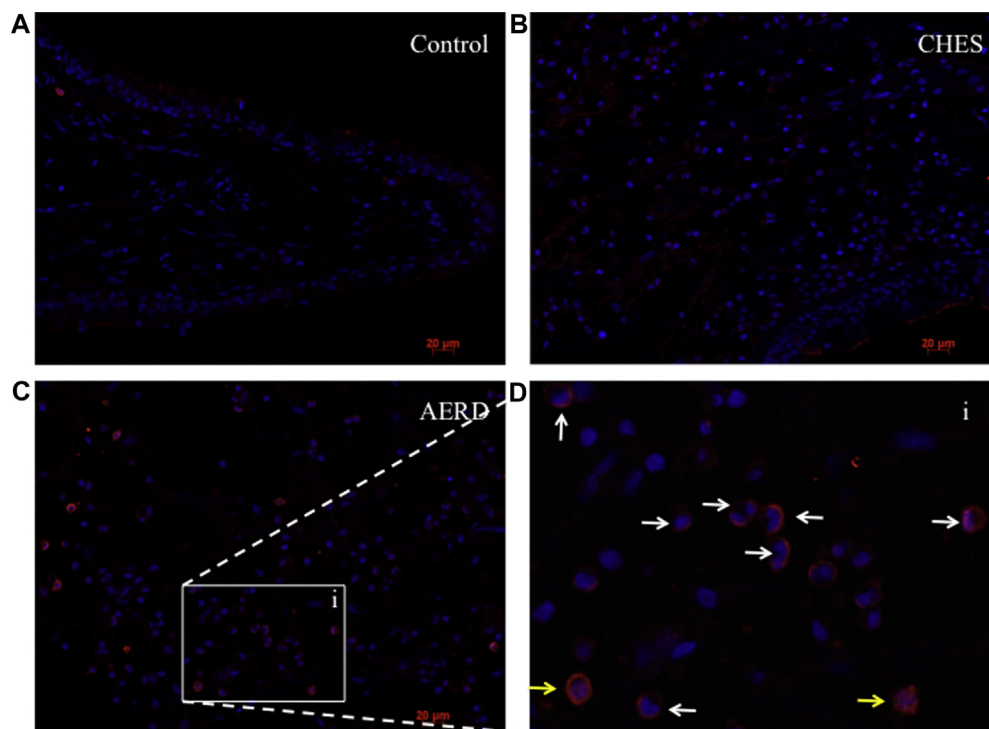


FIG 2. Immunofluorescence for hPGDS in polyps from patients with AERD and patients with CHES. hPGDS staining of paraffin-embedded sinus tissue using a primary antibody directed against hPGDS and an allophycocyanin-labeled secondary antibody (red) with 4',6-diamidino-2-phenylindole nuclear stain (blue). **A**, Control tissue. **B**, Tissue from a patient with CHES. **C**, Tissue from a patient with AERD. **D**, Insert (i) of tissue from a patient with AERD showing a close-up view. White arrows indicate eosinophils, and yellow arrows indicate mononuclear cells.

cell tryptase was highest in control tissue (13.6 ± 1.6 cells/hpf), followed by tissue from patients with CHES (11.1 ± 1.5 cells/hpf) and patients with AERD (6.8 ± 2.3 cells/hpf). These results support the immunofluorescence data showing eosinophils as being the most numerous cells expressing hPGDS in polyp tissue.

Expression of hPGDS from eosinophils

Eosinophils isolated from peripheral blood of patients with AERD ($n = 14$) were examined for expression of hPGDS mRNA and found to have increased levels ($P < .02$) compared with those in healthy subjects (3.48-fold higher, $n = 13$) or those with asthma (2.55-fold higher, $n = 12$; Fig 3, A). Protein expression for hPGDS was confirmed by means of Western hybridization (Fig 3, B), with higher levels observed in cells from the patients with AERD. Semiquantitative analysis verified significantly higher levels ($P < .03$) of hPGDS protein in eosinophils from patients with AERD compared with those from patients with asthma or control subjects without asthma (Fig 3, C).

Release of PGD₂ from eosinophils after aspirin activation

We have previously reported on the ability of aspirin to activate eosinophils directly to release mediators²⁰; however, we did not determine whether PGD₂ was released under these conditions. Eosinophils were purified from peripheral blood of healthy subjects ($n = 7$), asthmatic patients ($n = 6$), and patients with AERD ($n = 9$) and stimulated with various concentrations of

LysASA. Supernatants were collected, and PGD₂ levels were measured by means of ELISA. LysASA stimulated a dose-dependent release of PGD₂ from all groups (Fig 4), but the highest PGD₂ levels measured were from eosinophils from patients with AERD ($P < .005$). There were no between-group differences in PGD₂ release.

The ability of tissue eosinophils to also release PGD₂ was confirmed in a small number of samples by purifying eosinophils from sinus tissue and stimulating them *ex vivo* with LysASA. Similar to blood eosinophils, eosinophils from the tissue of patients with AERD demonstrated similar trends, with higher spontaneous levels of PGD₂ release ($1513 \text{ pg}/10^5$ in eosinophils from patients with AERD vs $826 \text{ pg}/10^5$ in eosinophils from patients with CHES) and higher levels of PGD₂ release after stimulation with 10 mmol/L LysASA ($1941 \text{ pg}/10^5$ in eosinophils from patients with AERD vs $1700 \text{ pg}/10^5$ in eosinophils from patients with CHES).

Influence of IFN- γ on hPGDS expression and PGD₂ release after eosinophil differentiation

Eosinophils were derived from CD34⁺ hematopoietic stem cells, as described, with or without the additional presence of IFN- γ . Expression of mRNA for hPGDS was significantly increased 3.75-fold ($P = .03$, $n = 12$) when progenitors were coincubated with IFN- γ (0.059 ± 0.023 without IFN- γ to 0.220 ± 0.072 with IFN- γ). Having demonstrated an increase in hPGDS levels, we investigated whether this would result in

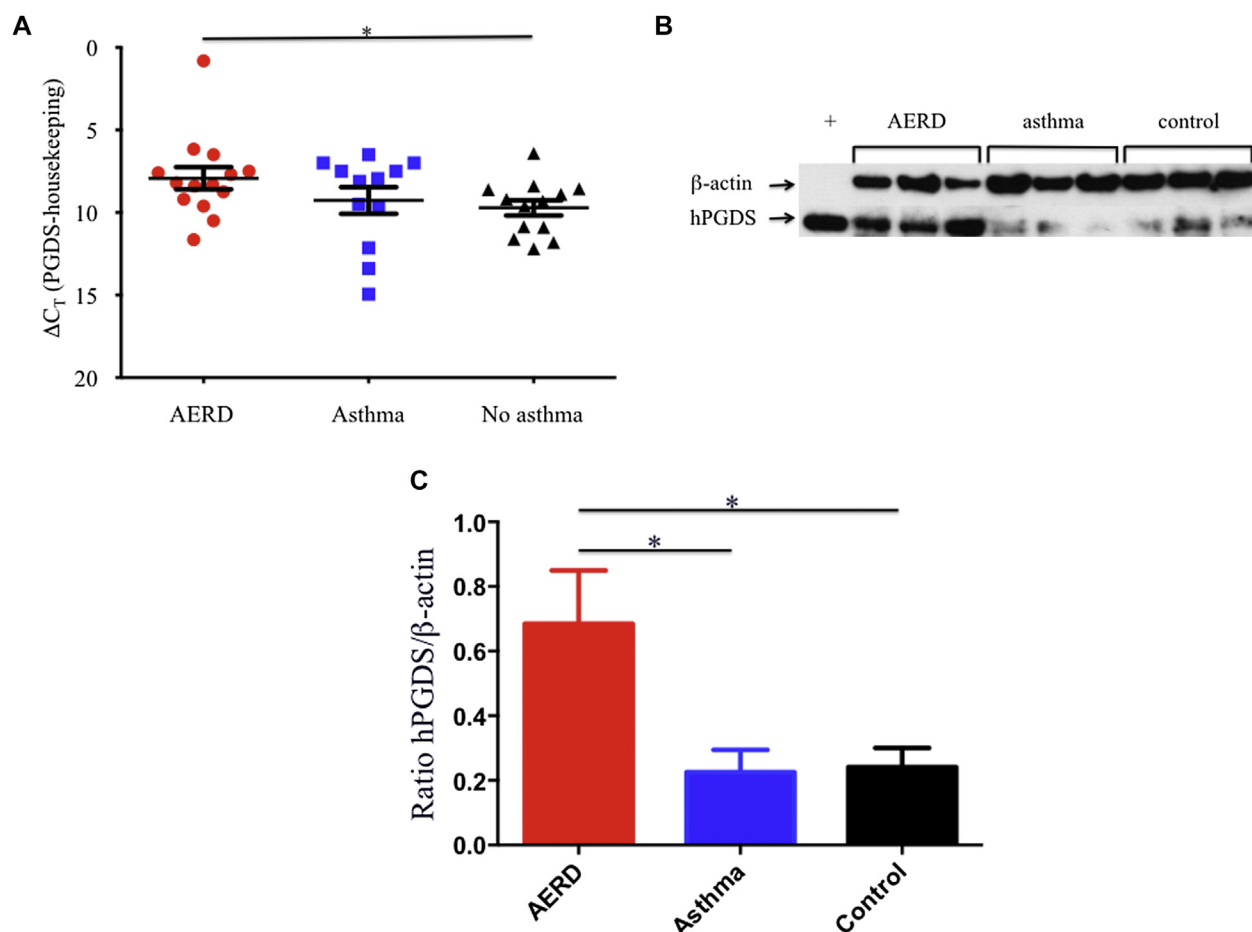


FIG 3. hPGDS expression in eosinophils. After separation of blood by using Ficoll-Hypaque density centrifugation, eosinophils were enriched with magnetic affinity column purification. **A**, Transcript levels of hPGDS were quantified by using PCR with SYBR Green detection. Data (mean $\pm$ SEM) reflect relative expression of each gene in comparison with the housekeeping gene EF1 α (Δ C_T). Control samples (n = 13) are depicted as *black triangles*, asthma samples (n = 12) as *blue squares*, and AERD samples (n = 14) as *red circles*. **B**, Measurement of hPGDS protein by means of Western hybridization. Eosinophils from 3 control subjects, asthmatic patients, and patients with AERD were collected and electrophoresed on a denaturing polyacrylamide gel and probed with rabbit antibody directed against hPGDS and β -actin as a loading control. **C**, Semiquantitative analysis of hPGDS protein levels (AERD, n = 9; asthma, n = 7; and control, n = 10). **P* < .02 compared with control values.

greater PGD₂ release. Spontaneous levels of PGD₂ release (Fig 5) were significantly greater in eosinophils (n = 10) cultured with IFN- γ than those without (*P* < .006), and LysASA stimulation showed a dose-dependent increase in the levels of PGD₂ released (*P* < .004) in both stimulated and unstimulated conditions.

Mechanism of PGD₂ release after aspirin activation of eosinophils

An unanswered question pertained to how PGD₂ was made after activation with LysASA, which should irreversibly inhibit COX-1. To test whether it was through a COX-1-dependent or COX-1-independent pathways, eosinophils were incubated with ibuprofen (10 mmol/L) or SC-560 (0.1 mmol/L), both of which are COX-1 inhibitors, for 30 minutes. Cells were washed to remove any excess compound and stimulated with varying doses of LysASA for an additional 30 minutes. Measurement of PGD₂ levels in the supernatant demonstrated that COX-1 inhibition did

not prevent PGD₂ release after LysASA stimulation (*P* < .05, Fig 6).

DISCUSSION

AERD is a disorder characterized by severe eosinophilic infiltrate into the sinus and respiratory tract, baseline overproduction of CysLTs, and unique sensitivity to leukotriene (LT) E₄ after aspirin ingestion.^{1,15,24,25} Expression of leukotriene C₄ synthase in eosinophils drives much of the overproduction of CysLTs,²⁴ with additional components driven by the activation of mast cells^{3,5,26,27} and platelet-adherent neutrophils.²⁸

Although contributing to many of the untoward effects of AERD, as evinced by the beneficial effects of LT modifiers,²⁹ increasing attention has been given to contributions from PGD₂. PGD₂ and its metabolites are higher at baseline in patients with AERD subjects in comparison with aspirin-tolerant subjects.^{30,31} Among the patients with AERD, there exists a subgroup with

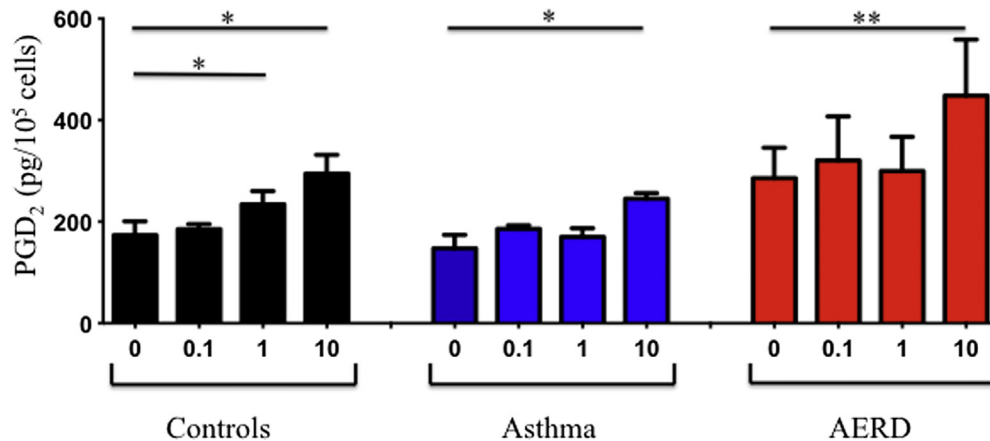


FIG 4. PGD₂ release from aspirin-activated eosinophils. Eosinophils isolated from peripheral blood of control subjects (black, n = 7), asthmatic patients (blue, n = 6), and patients with AERD (red, n = 9) were activated with various doses of LysASA for 30 minutes. Supernatants were collected, and PGD₂ levels were quantified (in picograms per 10⁵ cells). Data are presented as means ± SEMs. **P* < .02 or ***P* < .005 in comparison with unstimulated cells.

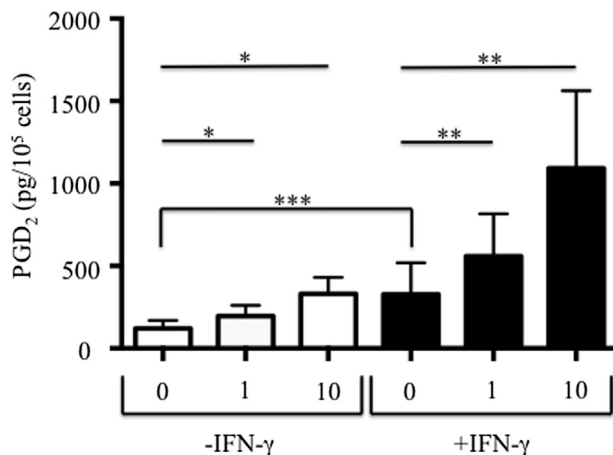


FIG 5. PGD₂ secretion by *in vitro*-differentiated eosinophils in the presence of IFN-γ. CD34⁺ enriched hematopoietic stem cells were differentiated into eosinophils with or without the additional presence of IFN-γ. Newly generated eosinophils were activated for 30 minutes, supernatants were collected, and PGD₂ levels were quantified (in picograms per 10⁵ cells). Data are presented as means ± SEMs (n = 10). **P* < .004 compared with 0-IFN-γ, ***P* < .004 compared with 0+IFN-γ, and ****P* < .006 compared to 0-IFN-γ.

particularly enhanced overproduction and release of PGD₂ during aspirin challenge that correlates with the severity of AERD and predicts failure of aspirin desensitization.⁶ In addition to defining a more severe subgroup, PGD₂ likely contributes to the severity of AERD through its myriad of biological activities that include inducing vasodilation and vascular leakage, as well as bronchoconstriction. Many stromal and inflammatory cells found in tissue from patients with AERD express receptors for PGD₂, including eosinophils, basophils, mast cells, epithelium, endothelium, and dendritic cells.³²⁻³⁵ In addition to producing PGD₂, eosinophils undergo chemotaxis in response to PGD₂, which thereby worsens tissue eosinophilia.^{32,34} In a murine study PGD₂ in conjunction with CCL11 enhanced eosinophil LTC₄ production.³⁶ PGD₂ also activates basophils driving upregulated expression of CD203c and CD11b.³⁷ On epithelium, PGD₂ acts to alter their

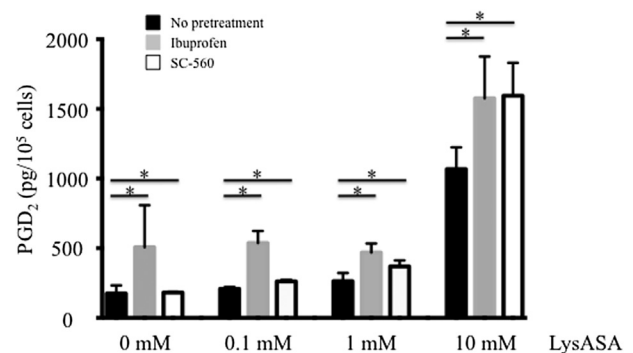


FIG 6. COX-1 inhibition does not prevent aspirin-induced PGD₂ release. Eosinophils isolated from peripheral blood were incubated with or without the COX-1 inhibitors, ibuprofen, or SC-560 for 30 minutes. Cells were washed and incubated for 30 minutes with increasing concentrations of LysASA, and supernatants were collected for measurement of PGD₂. Data are presented as means ± SEMs (n = 3). *Not significant in comparison with no pretreatment cells.

differentiation.³⁵ Perhaps the most important potential effect of PGD₂ in patients with allergic inflammation is through its ability to recruit, activate, and promote cytokine secretion from both T_H2-like lymphocytes and type 2 innate lymphoid cells.⁸⁻¹¹

PGD₂ is primarily considered a mast cell-specific product; however, it has been reported that eosinophils can both express hPGDS and release PGD₂.¹⁷ Our studies and those of others demonstrate that eosinophils are more prevalent than mast cells in tissue from patients with AERD.^{12,16} Thus even if on a per-cell basis mast cells do produce more PGD₂ than eosinophils, it is likely that eosinophils might be the more important source of this arachidonate metabolite in patients with AERD. This prompted us to examine the role of PGD₂ production by eosinophils in patients with AERD.

mRNA was extracted and investigated for hPGDS expression to answer the basic question regarding the ability of PGD₂ to be produced by polyps. Similar to previous reports,^{18,19} we found expression of hPGDS in NPs of patients with CRS (Fig 1). Importantly, the levels of hPGDS transcripts were significantly

higher in patients with AERD (Fig 1). In addition, based on immunofluorescence staining of polyp tissue from patients with AERD, hPGDS protein expression was particularly evident in eosinophils, although with additional expression by a mononuclear cell population, presumably mast cells (Fig 2), and this confirmed with antitrypsin staining. We did observe hPGDS⁺ cells in the CHES group, with again both eosinophils and mononuclear cells staining positive; however, the overall numbers were less than in patients with AERD (see Fig E2, A). Only occasional hPGDS-expressing cells were observed in control tissue, and this was confined to mononuclear (presumably mast) cells. Of interest was the finding that mast cell numbers were highest in control tissue followed by tissue from patients with CHES or AERD. Thus even though mast cell numbers were higher in the control tissue, they did not express high hPGDS levels; prominent expression only became evident as the disease state increased in severity.

We next analyzed hPGDS expression by peripheral blood eosinophils from patients with AERD, asthmatic patients, and control subjects. At both the mRNA and protein levels, the highest expression was observed in eosinophils from patients with AERD (Fig 3). We have published that eosinophils can be directly activated by aspirin to release CysLTs and ECP.²⁰ This prompted us to query whether aspirin activation would also stimulate PGD₂ release. In a dose-dependent manner aspirin triggered PGD₂ release (Fig 4), with the highest levels released from eosinophils from patients with AERD. In addition to levels of expression of PGD₂ synthase mRNA transcripts and protein, the capacity for a given eosinophil to secrete PGD₂ almost certainly will be further influenced by posttranslation mechanisms, including the responsiveness of the cell to activation pathways, such as those signaled by aspirin itself. In addition, given the results of our studies¹² and others^{14,15} that eosinophils are up to 10-fold more prevalent in patients with AERD than in sinus and lung tissue from aspirin-tolerant subjects, eosinophil numbers are likely to be a prominent determinant *in vivo* of the capacity for PGD₂ production.

Previously, we demonstrated a role for IFN- γ in accelerating the terminal maturation of eosinophils, as indicated by their surface expression of CCR3 and Siglec-8 and release of eosinophil-derived products.²¹ These studies also demonstrated that IFN- γ was uniquely able to increase expression of leukotriene C₄ synthase, an effect not mediated by any of the T_H2 signature cytokines studied,²¹ and we further showed that this upregulation led to increased secretion of CysLTs when cells were stimulated with aspirin.²⁰ We speculated that a similar result would be observed with hPGDS expression and PGD₂ secretion from IFN- γ -matured progenitor cells. Our data demonstrated a significant increase in IFN- γ -driven hPGDS expression in eosinophil progenitors. In the absence of IFN- γ , the progenitors displayed a dose-dependent increase in PGD₂ release after aspirin stimulation, and levels of PGD₂ released were augmented when the cells had matured in the presence of IFN- γ (Fig 5). AERD is a mixed T_H1/T_H2 phenotype with robust upregulation of IFN- γ .^{21,38} This unique environment, which defines the tissue from patients with AERD in which eosinophil progenitors are expressed and matured,³⁹⁻⁴¹ generates cells primed for release of mediators that will exacerbate symptoms. Increased production and release of both CysLTs and PGD₂ explains in part how ingestion of aspirin can lead to bronchoconstriction and airway hyperreactivity. It is interesting to speculate that the group of

subjects reported by Cahill et al⁶ who cannot be desensitized to aspirin because of overproduction of PGD₂ might have progenitor and mature eosinophils that express high levels of hPGDS, resulting in exaggerated PGD₂ release when the cells are stimulated. These results again suggest that IFN- γ or signal transducer and activator of transcription 1 might be attractive therapeutic targets in patients with AERD because they would target early progenitor cells, preventing the conversion to cells capable of synthesizing large quantities of arachidonic metabolites.

Although it might seem paradoxical that ingestion of COX inhibitors drives increased production of the COX product PGD₂, this can be readily explained by the superseding influences of the direct activating effects of aspirin (and other COX inhibitors) on eosinophils and mast cells^{5,20,42} combined with the robust influences of releasing these cells from the constraints provided by the anti-inflammatory PGE₂.⁴³⁻⁴⁵ It is intriguing to speculate that some of the beneficial influences of aspirin desensitization observed with long-term administration might be linked to inhibition of PGD₂ expression. Reactions to aspirin and nonselective nonsteroidal anti-inflammatory drugs in patients with AERD reflect COX-1 inhibition, whereas PG production by eosinophils (and mast cells) reflects the biological activity of COX-2.^{16,46} Therefore to address whether the mechanism of PGD₂ production after aspirin stimulation was driven by COX-1 inhibition, we performed experiments in which eosinophils were preincubated with COX-1 inhibitors before aspirin activation and observed no reduction in (COX-2-mediated) PGD₂ release (Fig 6). Thus the mechanism of aspirin-induced PGD₂ secretion is driven by one of the alternative (COX-independent) pathways modulated by aspirin.⁴⁷

Despite the use of LT pathway inhibitors and aspirin desensitization, AERD remains a disease with high morbidity.⁴⁸ Although IL-5 is not a distinguishing feature of AERD when compared with patients with aspirin-tolerant chronic sinusitis, it is clearly increased compared with that in healthy control subjects and those with chronic sinusitis without polyps, suggesting it might yet be a useful target for eosinophilic sinusitis.⁴⁹ Multiple biotherapeutic agents are in development or have clinical approval that target the eosinophil, including anti-IL-5 mAbs (mepolizumab and reslizumab) and anti-IL-5 receptor antibodies (benralizumab).⁵⁰⁻⁵² Given the particularly robust expression of infiltrating eosinophils in tissue from patients with AERD and the potential importance of eosinophil-derived mediators, including PGD₂, in driving the AERD phenotype, our results further support the concept that these agents are likely to be uniquely effective in patients with severe asthma with AERD.

In summary, our studies demonstrate that eosinophils can synthesize PGD₂ and that, in patients with AERD, eosinophils have a greater capacity to produce and secrete PGD₂ after aspirin stimulation than those from healthy subjects. In an environment rich in IFN- γ , eosinophil progenitor cells express increased levels of hPGDS and are hyperresponsive to aspirin stimulation. Although on a per-cell basis mast cells can synthesize more PGD₂, in patients with AERD, there is a massive influx of eosinophils into the sinus and lung tissue making the contribution of PGD₂ by eosinophils likely a more substantial contributor of the total amount released. Targeting PGD₂ synthesis or binding to its receptors presents an opportunity for therapeutic intervention in patients with this difficult-to-treat disease, and

indeed, this might underlie some of the beneficial effects observed after aspirin desensitization.

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Clinical implications: Demonstration of eosinophils as a source of PGD₂, which has been shown to be involved in the presence and severity of AERD, identifies a new target for therapeutic intervention.

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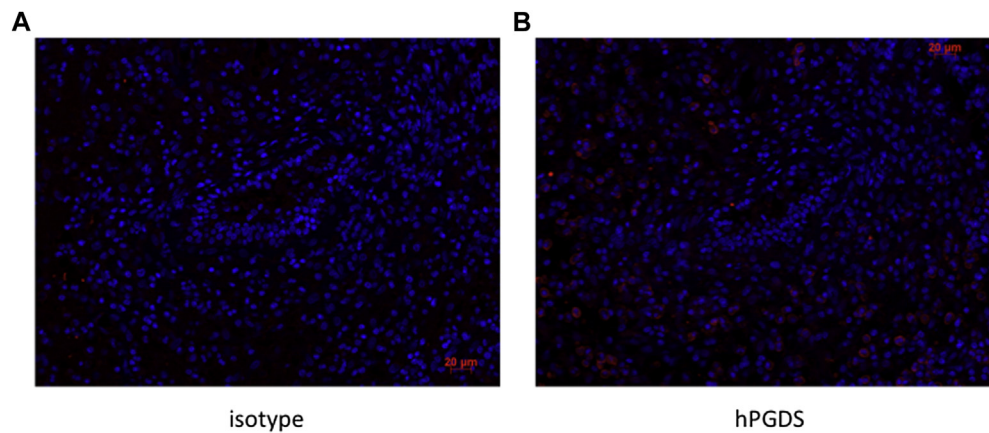


FIG E1. Isotype control of immunofluorescence for hPGDS. We performed immunofluorescence staining of polyp tissue, as described in the [Methods](#) section. **A**, Tissue from patients with AERD was incubated with polyclonal rabbit sera and allophycocyanin-labeled secondary antibody. **B**, Tissue from patients with AERD was incubated with rabbit anti-hPGDS primary and allophycocyanin-labeled secondary antibodies.

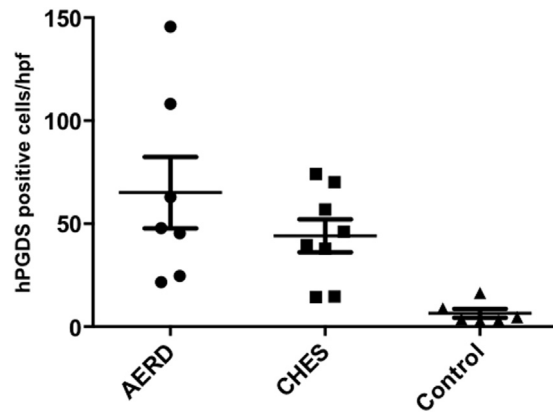


FIG E2. Quantification of hPGDS⁺ cells detected by means of immunofluorescence. Images were generated as described and analyzed by counting hPGDS⁺ and total cells (with the exclusion of epithelial and glandular cells). A *dot plot* showing positive hPGDS⁺ cells/hpf for tissue samples from patients with AERD (n = 7), patients with CHES (n = 8), and control subjects (n = 6).

TABLE E1. Subjects' characteristics

	Control subjects (n = 15)	Asthmatic patients (n = 18)	Patients with AERD (n = 10)
Female sex (%)	9 (60)	14 (78)	7 (70)
Age (y), mean $\pm$ SEM	37 $\pm$ 4	20 $\pm$ 1	41 $\pm$ 4
Positive atopic status (%)	8 (53)	18 (100)	4 (40)