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# Eosinophil production of $PGD_2$ in Aspirin-Exacerbated Respiratory Disease

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## Abstract

**Background—**Aspirin-exacerbated respiratory disease (AERD) differs from aspirin tolerant disease due in part to eosinophilic tissue infiltration and over-expression of arachidonic acid metabolic pathway components that lead to enhanced secretion of cysteinyl leukotrienes (CysLT) and prostaglandin $D_2$ ($PGD_2$) observed constitutively and, paradoxically, in response to aspirin and other cyclooxygenase inhibitors. We have previously demonstrated the capacity of interferon (IFN)-γ to drive CysLT expression and response.

**Objective—**We investigated eosinophils as a source for $PGD_2$ production in AERD.

**Methods—**Eosinophils were enriched from tissue and peripheral blood obtained from control, aspirin tolerant, and AERD subjects. mRNA was extracted and evaluated for expression of hematopoietic PGD synthase (hPGDS). Expression of hPGDS protein was confirmed with western hybridization and immunofluorescence staining. Cells were stimulated with aspirin and secretion of $PGD_2$ quantified. $CD34^+$ progenitor cells were isolated and matured into eosinophils in the presence or absence of IFN-γ and hPGDS mRNA and $PGD_2$ release measured.

**Results—**Gene expression analysis revealed that eosinophils from AERD tissue and blood display increased levels of hPGDS compared with asthmatic and control samples. Western hybridization confirmed the increase in hPGDS mRNA translated to increased protein expression.

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Immunofluorescence confirmed mast cells and eosinophils from AERD and asthmatic tissue demonstrated hPGDS expression with higher levels in AERD eosinophils. Incubation of eosinophils from blood and tissue with aspirin stimulated $PGD_2$ release. IFN-γ-matured eosinophil progenitors showed enhanced hPGDS expression and increased levels of $PGD_2$ release at baseline and following stimulation with aspirin.

**Conclusions—**In addition to mast cells, eosinophils represent an important source of $PGD_2$ in AERD and identify a new target for therapeutic intervention.

**Clinical Implications—**Demonstration of eosinophils as a source of $PGD_2$, that has been shown to be involved in the presence and severity of AERD, identifies a new target for therapeutic intervention.

### Keywords

aspirin exacerbated respiratory disease; aspirin tolerant asthma; chronic sinusitis; cytokines; eosinophils; prostaglandin $D_2$; nasal polyps

## Introduction

Aspirin-exacerbated respiratory disease (AERD) is a disease of the upper (chronic rhinosinusitis (CRS)/nasal polyposis (NP)) and (usually) the lower (asthma) airways that differs from aspirin tolerant asthma/chronic sinusitis by the unique sensitivity to aspirin and other non-selective cyclooxygenase (COX)-1 inhibitors. AERD comprises as many as 7% of adult-onset asthmatics and up to 12-14% of adult asthmatics with severe asthma [1, 2]. During aspirin reactions, many mediators are released including cysteinyl leukotrienes (CysLT), tryptase, ECP and prostaglandin $D_2$ ($PGD_2$) suggesting both mast cell and eosinophil activation [3-5].

In AERD, baseline levels of $PGD_2$ and its metabolites are higher in the blood than in subjects who are aspirin tolerant and following ingestion of aspirin these levels further increase in AERD subjects [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 this group that fails desensitization is distinguished by their constitutive overproduction and aspirin-stimulated release of $PGD_2$ that correlates with the severity of airflow obstruction [6]. In addition to defining a more severe subgroup that fails aspirin desensitization, $PGD_2$ likely contributes to the severity through its myriad of biological activities that includes inducing vasodilation and vascular leakage, bronchoconstriction, and the recruitment and activation of basophils, eosinophils, dendritic cells, and both Th2-like lymphocytes and type 2 innate lymphoid cells [8-11].

While 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 others [14-16] that tissue from AERD subjects express ~3-fold greater numbers of tissue eosinophils and eosinophilcationic protein (ECP) compared to aspirin tolerant subjects. $PGD_2$ has historically been thought of as a mast cell product, however eosinophils can express hematopoietic prostaglandin $D_2$ synthase (hPGDS), the enzyme responsible for $PGD_2$

synthesis, and eosinophils do secrete $PGD_2$ [17]. Given that eosinophil expression greatly exceeds that of mast cells in AERD it is reasonable to speculate that eosinophils maybe also be an important source of $PGD_2$. Expression of hPGDS has been observed in NP of CRS patients [18] and one report suggested that a subpopulation of eosinophils in the NP were one source of the enzyme [19].

The current studies were therefore performed to assess the production of $PGD_2$ by eosinophils in AERD. In particular, we investigated increased expression of hPGDS in NP of AERD subjects 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]. We therefore investigated $PGD_2$ release when AERD eosinophils were stimulated with aspirin. In addition, we have demonstrated that interferon (IFN)-γ uniquely contributes to the development and differentiation of eosinophils, enhancing their ability to produce and release mediators including cysteinyl leukotrienes and eosinophil derived neurotoxin [21]. We therefore also queried whether a similar result would be observed with expression of hPGDS and subsequent $PGD_2$ release from eosinophil progenitors matured in the presence of this cytokine.

## Methods

### Subjects

Nasal polyp 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 (e.g. orbital decompression, cerebrospinal fluid leak repair, or transphenoidal pituitary surgery). Depending upon 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 upon the presence of ≥5 eosinophils/400x high powered field [1]. AERD was defined by a compelling history involving a hypersensitivity reaction within 2-3 hrs of ingestion of either aspirin or another non-steroidal anti-inflammatory drug. 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 AERD, 12 asthmatic and 15 non-asthmatic controls were enrolled though all subjects were not involved with every experiment: see individual figures for exact number. Subject characteristics for those involved in the blood draw are show in the online supplement (Table E1).

### Immunofluorescence and anti-tryptase 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 min in citrate buffer (Abcam; Cambridge, MA). Slides were washed and blocked using 1% bovine serum albumin, 10% goat serum (Sigma, St.

Louis, MO) and Fc Block 1 μg (BD Pharmingen; Sparks, MD) for 2 hrs. Specific staining for hPGDS was performed using a 1:150 dilution of a rabbit anti-human hPGDS antibody (Abcam ab89709) for 16 hrs 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 DAPI (4', 6-diamidino-2-phenylindole, Sigma) for 30 min at room temperature. Samples were washed in phosphate buffered saline and aqueous mounted with VectaMount AQ (Vector Laboratories; Burlington, CA). The samples were analyzed using a Zeiss AxioImager Z2 equipped with Apotome for optical sectioning (Zeiss; Thornwood, NY) and hPGDS positive cells scored in a blinded fashion. For anti-tryptase staining, rehydrated slides were treated with 3% peroxidase for 5 min followed by antigen retrieval (Dako target retrieval solution pH 9 (Dako, 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 using the Envision+System-HRP (AEC) (Dako) with labeled polymer added for 30 min and chromogen for 5 min followed by counterstaining with Gills hematoxylin (Sigma) for 3 min. Tryptase positive cells were scored in a blinded fashion taking the average of 10 fields per slide.

### Eosinophil Isolation

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

### Western hybridization of cellular extracts

Eosinophils were lysed and equal amounts of protein were added to 5X Laemmli Sample Buffer (GeneScript; Piscataway, NJ) and heated for 5 min at 95° C. Samples were electrophoresed on 12% TruPAGE precast gels (Sigma) and transferred to a polyvinylidene difluoride membrane (Millipore; Bilerica, MA) for 18 hrs at 40V. The membrane was blocked with TBST buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20) with 5% non-fat dry milk before addition of anti-hPGDS antibodies (Abcam). The target protein was visualized with the addition of the goat anti-rabbit horseradish peroxidase secondary antibody (Bio-Rad; San Jose, CA), after which blots were developed using the Immobilon Chemiluminescent kit (Millipore). After primary analysis, blots were stripped with 100 mM glycine and reprobed with an antibody to ß-actin. Analysis of blots was performed via densitometry (GS-800 Calibrated Densitometer (Bio-Rad)) using the ratio of hPGDS to ß-actin calculated with ImageQuant TL v2005 (Amersham Biosciences, Pittsburgh, PA).

### Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed on NP tissue, peripheral blood eosinophils and on newly differentiated eosinophils for hPGDS. For the NP studies, polyps were minced and digested with Accutase (Innovative Cell Technologies, San Diego, CA) for 1 hr at 37 °C and the leukocyte-containing fraction was collected by passing the cell suspension through a 70 μm nylon mesh strainer (BD Falcon, Bedford, MA). Eosinophils were enriched using magnetic affinity

column purification with positive selection for CD15 followed by negative selection for CD16 (Miltenyi Biotec; San Diego, CA) and were >90% pure and viable as measured by flow cytometry. Total RNA was extracted using TRI® reagent (Sigma). Conversion of mRNA to cDNA was performed using a Taqman Reverse Transcription kit (Roche, Branchburg, NJ). Total RNA (200 ng) was added to each reaction along with oligo dT primers, 5.5 mM $MgCl_2$, 2 mM dNTPs, RNasin, and reverse transcriptase. Reactions went through 10 min at 25° C, 30 min at 48° C and 5 min at 95° C in a Bio-Rad iCycler thermocycler (Bio-Rad). The PCR mix consisted of Sensimix (Bioline; Taunton, MA), cDNA and 200 μM of each primer. Data were analyzed as the change in $C_T$ of each cytokine transcript in comparison to either EF1α or ß-actin (as appropriate based upon concordance of their $C_T$ with that of the gene of interest). Primers for EF1α were purchased (SABiosciences; Frederick, MD). The primers for hPGDS were: sense 5'-GGGCAGAGAAAAACAAGATGT-3' and antisense 5'-CCCCCCTAAATATGTGTCCAAG-3' (Integrated DNA Technologies, Inc., Coralville, IA).

## Eosinophil progenitor activation

Peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Hypaque density centrifugation from blood obtained from 10 healthy volunteers. $CD34^+$ cells were purified (>90%) from PBMCs using positive magnetic affinity column purification ($CD34^+$; Miltenyi Biotec) and eosinophil progenitors were derived using the technique of Hudson et al. [23] by culturing purified $CD34^+$ cells in complete medium (RPMI1640 and 10% FBS) supplemented with stem cell factor (SCF; 25 ng/ml; BD Biosciences), thymopoietin (TPO; 25 ng/ml; R&D System, Minneapolis, MN), Fms-like tyrosine kinase 3(Flt3) ligand (25 ng/ml; BD Biosciences), IL-3 (25 ng/ml; BD Biosciences) and IL-5 (25 ng/ml; BD Biosciences) with or without interferon (IFN)-γ (20 ng/ml; BD Bioscience) for 3 days and then cultured for an additional 3 weeks with just the IL-3 and IL-5 (again ±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 flow cytometry, the cells were 3.1% dual positive for CCR3/Siglec-8 without IFN-γ and 17.7% dual positive for CCR3/Siglec-8 with IFN-γ. The cells were examined for expression of FcεRI and CD203c as markers of basophils and these markers were found on less than 3% of the cells. Morphologically the newly differentiated eosinophils had bi-lobed 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 mM) for 30 min. For studies involving pretreatment with COX-1 inhibitors, eosinophils were incubated for 30 min with either 10 mM ibuprofen sodium salt (Santa Cruz, Dallas, TX) or 0.1 mM SC-560 (Santa Cruz) after which cells were washed in PBS and stimulated with varying doses of LysASA for an additional 30 min. Supernatants were collected and assayed by enzyme immunoassay (EIA) for $PGD_2$ (Cayman; Ann Arbor, MI) according to the manufacturer's directions: lower limit of detection 55 pg/ml.

### Statistical analyses

Data were contrasted between unstimulated and stimulated cells using the Mann Whitney paired t-test for nonparametric data. Control, CHES and AERD cohorts were compared using nonparametric unpaired t-tests. Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA).

## Results

### Differential expression of hPGDS in sinus tissue

NP tissue from AERD (n=9) and CHES subjects (n=17) along with control sinus tissue (n=9) was digested and RNA extracted. Expression of hPGDS was measured by qPCR (Figure 1) and ΔCT for AERD was 4.34 and 4.33 cycles earlier than the housekeeping gene EF1α for CHES and control groups, respectively, reflecting that hPGDS transcript expression was ~20 fold higher ($p<0.02$) in the AERD group.

### Immunofluorescence staining of hPGDS in nasal polyps

To confirm the post-translational expression of hPGDS and to define the cellular source, we performed immunofluorescence staining on tissue samples from AERD, CHES and control tissue. Representative images are shown in Figure 2 and isotype control is displayed in supplementary Figure E1. Essentially no hPGDS staining was observed in control tissue, largely reflecting the absence of inflammatory cells (Figure 2A). Moderate levels of hPGDS were detected in CHES samples (Figure 2B) with the highest levels found in AERD tissue (Figure 2C). As shown in the insert (Figure 2D), most of these cells were identified as eosinophils by being granulocytes with bi-lobed nuclei (white arrows) or alternatively were mononuclear cells without visualizable granules, most likely mast cells (yellow arrows). The average number of hPGDS expressing cells per high-powered field (hpf) was determined (Figure E2). The highest numbers were found in AERD, with the majority of cells identified as eosinophils consistent with our previous studies [12]. To confirm that the majority of cells observed as staining positive for hPGDS were eosinophils, mast cell numbers in tissue sections were counted. Staining for mast cell tryptase was highest in control tissue 13.6±1.6 cells/hpf, followed by CHES 11.1±1.5 cells/hpf and 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 AERD subjects (n=14) were examined for expression of hPGDS mRNA and found to have elevated levels ($p<0.02$) compared to healthy individuals (3.48-fold higher: n=13) or those with asthma (2.55-fold higher: n=12) (Figure 3A). Protein expression for hPGDS was confirmed via Western hybridization (Figure 3B) with higher levels observed in cells from the AERD subjects. Semi-quantitative analysis verified significantly higher levels ($p<0.03$) of hPGDS protein in eosinophils from AERD subjects as compared to those with asthma or controls without asthma (Figure 3C).

### Release of $PGD_2$ from eosinophils following aspirin activation

We have previously reported on the ability of aspirin to activate eosinophils directly to release mediators [20], however we did not determine whether or not $PGD_2$ was released under these conditions. Eosinophils were purified from peripheral blood of healthy (n=7), asthmatic (n=6) and AERD subjects (n=9) and stimulated with various concentrations of LysASA. Supernatants were collected and $PGD_2$ measured by ELISA. LysASA stimulated a dose-dependent release of $PGD_2$ from all groups (Figure 4), but the highest levels of $PGD_2$ measured were from AERD eosinophils ($p<0.005$). There were no between group differences in $PGD_2$ release. The ability of tissue eosinophils to also release $PGD_2$ was confirmed in a small number of samples by purifying eosinophils from sinus tissue and stimulating them *ex vivo* with LysASA. Similar to the blood eosinophils, AERD tissue eosinophils demonstrated similar trends with higher spontaneous levels of $PGD_2$ release (1513 pg/$10^5$ AERD vs. 826 pg/$10^5$ CHES eosinophils) and higher levels of $PGD_2$ release following stimulation with 10 mM LysASA (1941 pg/$10^5$ AERD vs. 1700 pg/$10^5$ CHES eosinophils).

### Influence of IFN-γ on hPGDS expression and $PGD_2$ release following 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 ($p=0.03$; n=12) when progenitors were co-incubated with IFN-γ (0.059±0.023 without IFN-γ to 0.220±0.072 with IFN-γ). Having demonstrated an increase in hPGDS, we investigated whether this would result in greater $PGD_2$ release. Spontaneous levels of $PGD_2$ release (Figure 5) were significantly higher in eosinophils (n=10) cultured with IFN-γ than those without ($p<0.006$) and LysASA stimulation showed a dose-dependent increase in the levels of $PGD_2$ released ($p<0.004$) in both stimulated and unstimulated conditions.

### Mechanism of $PGD_2$ release following aspirin activation of eosinophils

An unanswered question pertained to how $PGD_2$ was being made following activation with LysASA, which should irreversibly inhibit COX-1. To test whether it was through a COX-1-dependent or -independent pathway, eosinophils were incubated with ibuprofen (10 mM) or SC-560 (0.1mM), both COX-1 inhibitors, for 30 min. Cells were washed to remove any excess compound and stimulated with varying doses of LysASA for an additional 30 min. Measurement of $PGD_2$ in the supernatant demonstrated that COX-1 inhibition failed to prevent $PGD_2$ release following LysASA stimulation (Figure 6; $p<0.05$).

## Discussion

AERD is a disorder characterized by severe eosinophilic infiltrate into the sinus and respiratory tract, a baseline over-production of CysLTs and a unique sensitivity to $LTE_4$ following ingestion of aspirin [1, 15, 24, 25]. Expression of $LTC_4S$ in eosinophils drives much of the over-production of CysLTs [24] with additional components driven by the activation of mast cells [3, 5, 26, 27] and platelet-adherent neutrophils [28]. While contributing to many of the untoward effects of AERD, as evinced by the beneficial effects of leukotriene modifiers [29],

increasing attention has been given to contributions from $PGD_2$. $PGD_2$ and its metabolites are higher at baseline in AERD subjects in comparison to aspirin tolerant individuals [30, 31]. Within the AERD patients, there exists a subgroup that has particularly enhanced overproduction and release of $PGD_2$ during aspirin challenges that correlates with the severity of AERD and predicts failure of aspirin desensitization [6]. In addition to defining a more severe subgroup, $PGD_2$ 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 AERD tissue express receptors for $PGD_2$, including eosinophils, basophils, mast cells, epithelium, endothelium, and dendritic cells [32-35]. In addition to producing $PGD_2$, eosinophils chemotax in response to $PGD_2$ which thereby worsen tissue eosinophilia [32, 34]. In a murine study $PGD_2$ in conjunction with CCL11 enhanced eosinophil $LTC_4$ production [36]. $PGD_2$ also activates basophils driving upregulated expression of CD203c and CD11b [37]. On epithelium, $PGD_2$ acts to alter their differentiation [35]. Perhaps the most important potential impact of $PGD_2$ in allergic inflammation is through its ability to recruit, activate, and promote cytokine secretion from both Th2-like lymphocytes and type 2 innate lymphoid cells [8, 910, 11].

$PGD_2$ is primarily considered to be a mast cell-specific product, however it has been reported that eosinophils can both express hPGDS and release $PGD_2$ [17]. Our studies and others demonstrate that eosinophils are more prevalent than mast cells in AERD tissue [12, 16]. Thus, even if on a per cell basis mast cells do produce more $PGD_2$ than eosinophils, it is likely that eosinophils may be the more important source of this arachidonate metabolite in AERD. This prompted us to examine the role of $PGD_2$ production by eosinophils in AERD.

To answer the basic question regarding the ability of $PGD_2$ to be produced by polyps, mRNA extracted and investigated for hPGDS expression. Similar to previous reports [18, 19] we found expression of hPGDS in NP of CRS patients (Figure 1). Importantly, the levels of hPGDS transcripts were significantly higher in the subjects with AERD (Figure 1). In addition, based on immunofluorescence staining of AERD polyp tissue, hPGDS protein expression was particularly evident in eosinophils, although with additional expression by a mononuclear cell population, presumably mast cells (Figure 2) and confirmed with anti-tryptase staining. We did observe hPGDS positive cells in the CHES group with again both eosinophils and mononuclear cells staining positive, however the overall numbers were less than in AERD (Figure E2A). 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 the control tissue followed by CHES and AERD. Thus, even though mast cells numbers were higher in the control tissue, they did not express high levels of hPGDS: prominent expression only became evident as the disease state increased in severity.

We next analyzed hPGDS expression by peripheral blood eosinophils from AERD, asthmatic and control subjects. On both the mRNA and protein levels, the highest levels of expression were observed in eosinophils from AERD subjects (Figure 3). We have published that eosinophils can be directly activated by aspirin to release CysLTs and ECP [20]. This prompted us to query if aspirin activation would also stimulate $PGD_2$ release. In a dose-dependent manner, aspirin triggered the release of $PGD_2$ (Figure 4) with the highest levels

released from AERD eosinophils. In addition to levels of expression of $PGD_2$ synthase mRNA transcripts and protein, the capacity for a given eosinophil to secrete $PGD_2$ almost certainly will be further influenced by post-translation mechanisms including the responsiveness of the cell to activation pathways, such as those signaled by aspirin itself. In addition, given the results of our studies [12] and others [14,15] that eosinophils are up to 10-fold more prevalent in AERD than aspirin tolerant sinus and lung tissue, eosinophil number is likely *in vivo* to be a prominent determinant of the capacity for $PGD_2$ 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 $LTC_4S$, an effect not mediated by any of the Th2 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 $PGD_2$ 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 $PGD_2$ release following aspirin stimulation and the level of $PGD_2$ released was augmented when the cells had been matured in the presence of IFN-γ (Figure 5. AERD is a mixed Th1/Th2 phenotype with robust upregulation of IFN-γ [21, 38]. This unique environment, that defines the AERD tissue in which eosinophil progenitors are expressed and mature [39-41], generates cells primed for release of mediators that will exacerbate symptoms. Increased production and release of both CysLTs and $PGD_2$ 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 al. that cannot be desensitized to aspirin due to overproduction of $PGD_2$ may have progenitor and mature eosinophils that express high levels of hPGDS resulting in exaggerated $PGD_2$ release when the cells are stimulated [6]. These results again suggest that IFN-γ or STAT1 may be attractive therapeutic targets in AERD as they would target early progenitor cells preventing the conversion to cells capable of synthesizing large quantities of arachidonic metabolites.

While it may seem paradoxical that ingestion of cyclooxygenase inhibitors drives increased production of the cyclooxygenase product $PGD_2$, 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 prostaglandin, $PGE_2$ [43-45]. It is intriguing to speculate the some of the beneficial influences of aspirin desensitization observed with long-term administration may be linked to inhibition of $PGD_2$ expression. Reactions to aspirin and non-selective NSAIDs in AERD reflect COX-1 inhibition, whereas prostaglandin production by eosinophils (and mast cells) reflects the biological activity of COX-2 [16, 46]. Therefore, to address whether the mechanism of $PGD_2$ production following aspirin stimulation was driven by COX-1 inhibition, we performed experiments in which eosinophils were preincubated with COX-1 inhibitors prior to aspirin activation and observed no reduction in (the COX-2-mediated) $PGD_2$ release (Figure 6). Thus, the mechanism of aspirin-induced $PGD_2$ secretion is driven by one of the alternative (COX-independent) pathways modulated by aspirin [47].

Despite the use of leukotriene pathway inhibitors and aspirin desensitization, AERD remains a disease with high morbidity [48]. While IL-5 is not distinguishing feature of AERD when compared to aspirin-tolerant chronic sinusitis, it is clearly elevated compared to healthy controls and those with chronic sinusitis without polyps, suggesting it may 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 monoclonal antibodies (mepolizumab and reslizumab) and anti-IL-5 receptor antibodies (benralizumab) [50-52]. Given the particularly robust expression of infiltrating eosinophils in AERD tissue and the potential importance of eosinophil-derived mediators – including $PGD_2$ – in driving the AERD phenotype, our results further support the concept that these agents are likely to be uniquely effective in severe asthmatics with AERD.

In summary, our studies demonstrate that eosinophils can synthesize $PGD_2$ and that, in AERD, eosinophils have a greater capacity to produce and secrete $PGD_2$ following aspirin stimulation than those from healthy individuals. In an environment rich in IFN-γ, eosinophil progenitor cells express increased levels of hPGDS and are hyperresponsive to aspirin stimulation. While on a per cell basis, mast cells can synthesize more $PGD_2$, in AERD there is a massive influx of eosinophils into the sinus and lung tissue making the contribution of $PGD_2$ by eosinophils likely a more substantial contributor of the total amount released. Targeting $PGD_2$ synthesis or binding to its receptors presents an opportunity for therapeutic intervention in this difficult to treat disease and, indeed, this may underlie some of the beneficial effects observed after aspirin desensitization.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **AERD** | aspirin exacerbated respiratory disease |
| **APC** | allophycocyanin |
| **CHES** | chronic hyperplastic eosinophilic sinusitis |
| **COX** | cyclooxygenase |
| **CRS** | chronic rhinosinusitis |
| **CysLT** | cysteinyl leukotriene |
| **DAPI** | 4’, 6-diamidino-2-phenylindole |
| **ECP** | eosinophil cationic protein |

| | |
|---|---|
| **EIA** | enzyme-linked immunoassay |
| **FBS** | fetal bovine serum |
| **Flt3L** | Fms-like tyrosine kinase 3 ligand |
| **hfp** | high-powered field |
| **hPGDS** | hematopoietic prostaglandin $D_2$ synthase |
| **IFN** | interferon |
| **IL** | interleukin |
| **LT** | leukotriene |
| **NP** | nasal polyps |
| **PBMC** | peripheral blood mononuclear cells |
| **PG** | prostaglandin |
| **PMA** | phyorbol myristate acetate |
| **qPCR** | quantitative polymerase chain reaction |
| **SCF** | stem cell factor |
| **TPO** | thymopoietin |

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## Capsule Summary

Increased levels of $PGD_2$ found in AERD come from both mast cells and eosinophils, the release of which likely contributes to the inflammation observed in AERD and severe upper and lower airways symptoms that develop upon ingestion of aspirin.

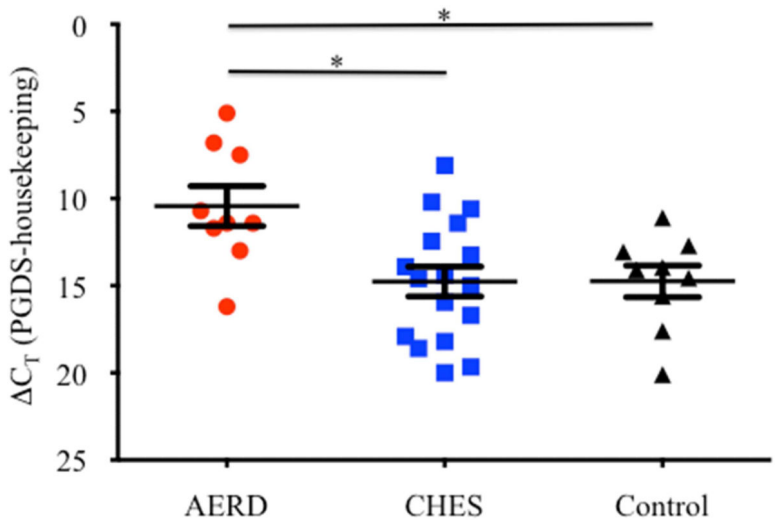


**Figure 1. hPGDS gene expression in sinus tissue by qPCR**

Tissue samples were homogenized following surgical removal and RNA isolated. Transcript levels of hPGDS were quantified using PCR with sybr-green detection. Data (mean±SEM) reflect relative expression of each gene in comparison to the housekeeping gene EF1α (ΔCT). Control samples (n=9) are depicted in black triangles, CHES (n=17) in blue squares and AERD (n=9) in red circles. *p<0.02 as compared to CHES and Control.

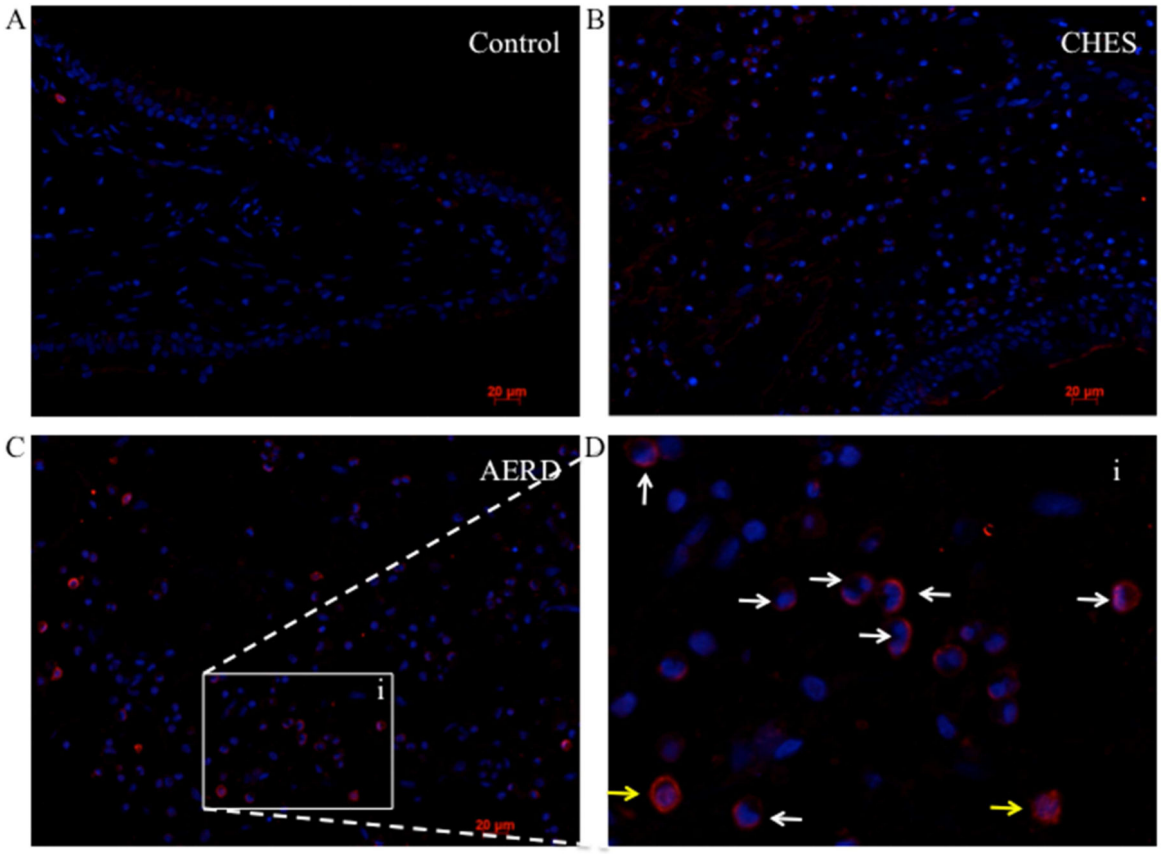


**Figure 2. Immunofluorescence for hPGDS in AERD and CHES polyps**

PGDS staining of paraffin embedded sinus tissue using a primary antibody directed against hPGDS and an APC-labeled secondary antibody (red) with DAPI nuclear stain (blue). A. Control tissue. B. CHES tissue. C. AERD tissue. D. insert (i) of AERD tissue showing a close-up view. White arrows indicate eosinophils and yellow arrows indicate mononuclear cells.

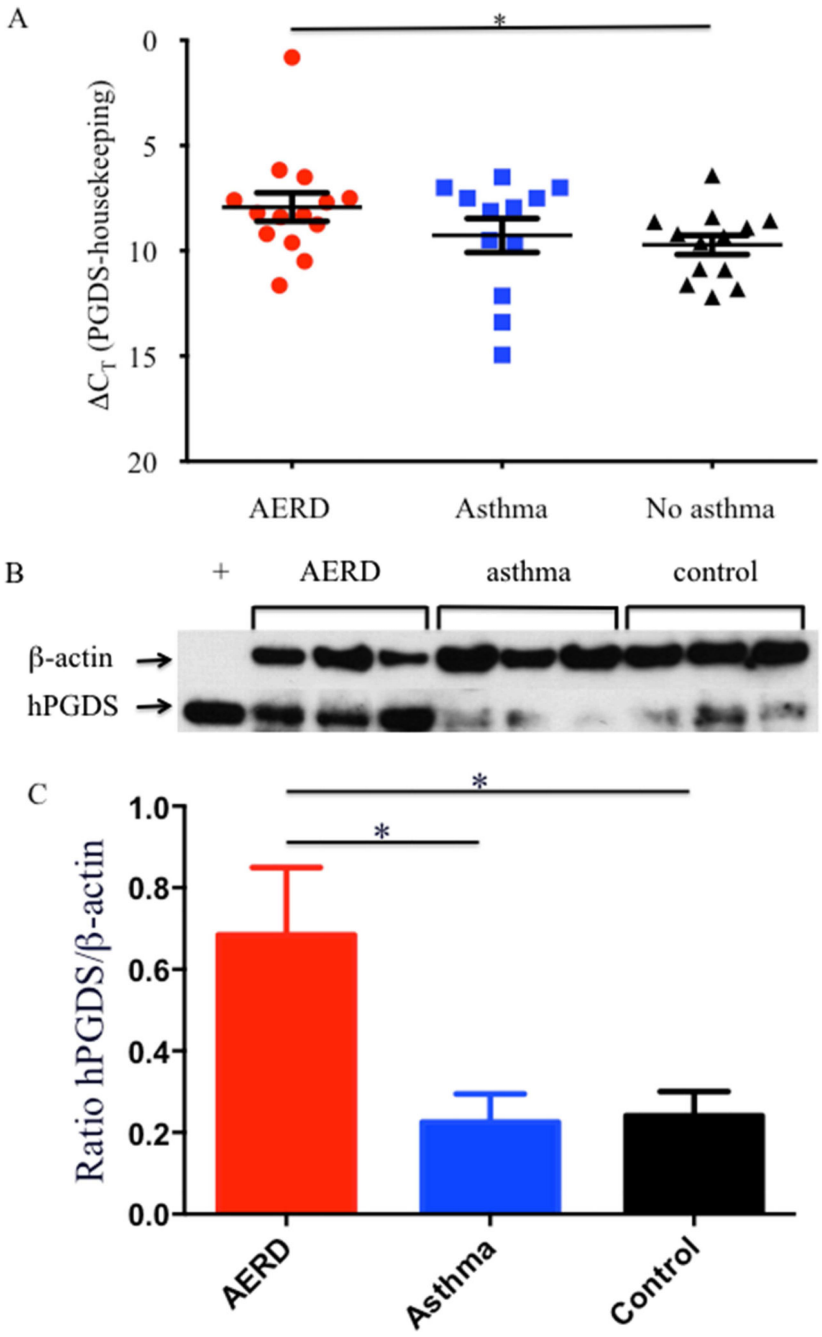


**Figure 3. hPGDS expression in eosinophils**

After separation of blood using Ficoll-Hypaque density centrifugation, eosinophils were enriched using magnetic affinity column purification. A. Transcript levels of hPGDS were quantified using PCR with sybr-green detection. Data (mean±SEM) reflect relative expression of each gene in comparison to the housekeeping gene EF1α (ΔCT). Control samples (n=13) are depicted in black triangles, Asthma (n=12) in blue squares and AERD (n=14) in red circles. B. Measurement of hPGDS protein by Western hybridization. Eosinophils from three control, asthmatic and AERD subjects were collected and electrophoresed on a denaturing polyacrylamide gel and probed with rabbit antibody

directed against hPGDS and ß-actin as a loading control. C. Semi-quantitative analysis of hPGDS protein levels. (AERD n=9, Asthma n=7, and Control n=10) *p<0.02 as compared to Control.

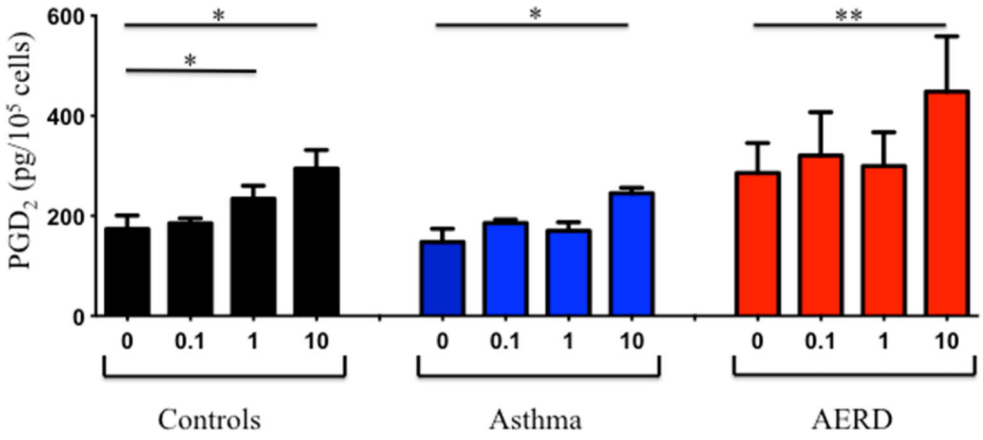


**Figure 4. $PGD_2$ release from aspirin-activated eosinophils**

Eosinophils isolated from peripheral blood of control (black: n=7), asthmatic (blue: n=6) and AERD (red: n=9) subjects were activated with various doses of LysASA for 30 minutes. Supernatants were collected and $PGD_2$ levels quantified (pg/$10^5$ cells). Data are presented as mean±SEM with *p<0.02 or **p<0.005 in comparison to unstimulated cells.

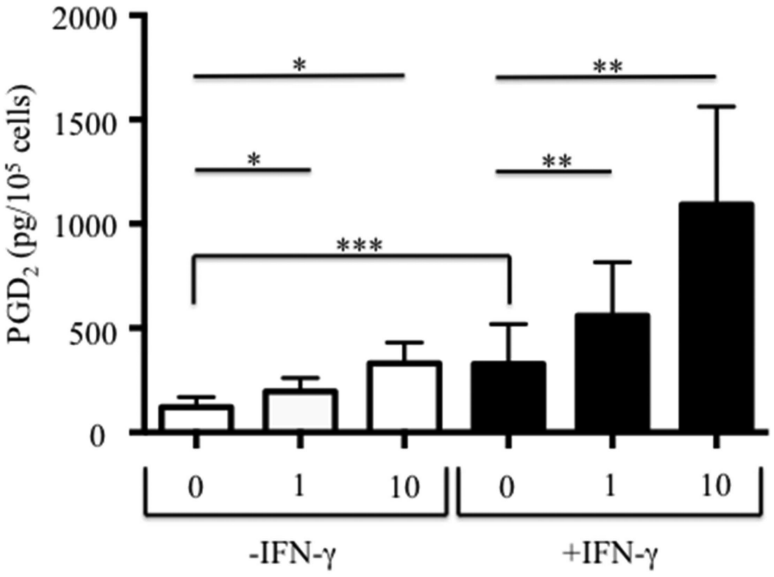


**Figure 5. $PGD_2$ secretion by *in vitro*-differentiated eosinophils in the presence of IFN-γ**

$CD34^+$-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 collected, and $PGD_2$ levels quantified ($pg/10^5$ cells). Data are presented as mean±SEM (n=10). *p<0.004 compared to 0-IFN-γ; **p<0.004 compared to 0+IFN-γ; ***p<0.006 compared to 0-IFN-γ.

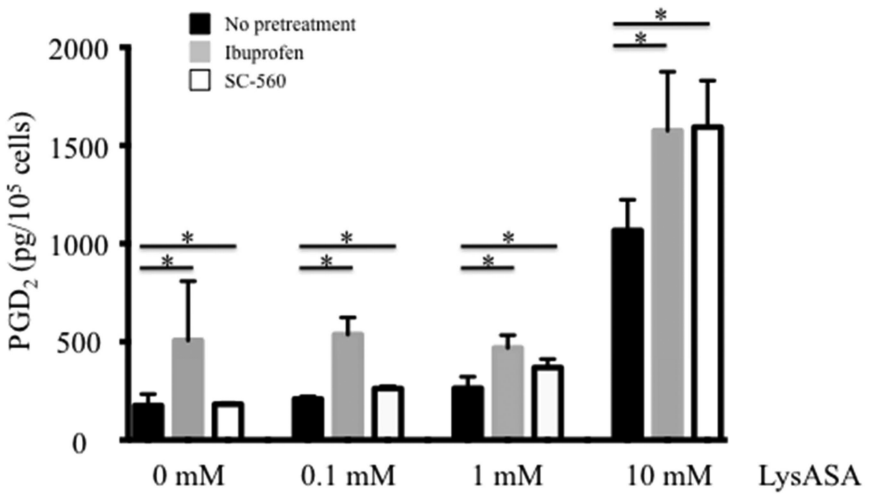


**Figure 6. COX-1 inhibition fails to prevent aspirin-induced $PGD_2$ release**

Eosinophils isolated from peripheral blood were incubated with or without the COX-1 inhibitors, ibuprofen or SC-560 for 30 min. Cells were washed and incubated for 30 min with increasing concentrations of LysASA and supernatants collected for measurement of $PGD_2$. Data are presented as mean±SEM (n=3) with *p=NS in comparison to no pretreatment cells.