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Prostaglandin E₂ resistance in granulocytes from patients with aspirin-exacerbated respiratory disease

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Abstract

Background—Aspirin-exacerbated respiratory disease (AERD) is an inflammatory condition of the respiratory tract and is characterized by overproduction of leukotrienes (LT) and large numbers of circulating granulocyte-platelet complexes. LT production can be suppressed by prostaglandin E₂ (PGE₂) and the cyclic AMP-dependent protein kinase A (PKA).

Objective—To determine if PGE₂-dependent control of LT production by granulocytes is dysregulated in AERD.

Methods—Granulocytes from well-characterized patients with and without AERD were activated ex vivo and subjected to a range of functional and biochemical analyses.

Results—Granulocytes from subjects with AERD generated more LTB₄ and cysteinyl LTs than did granulocytes from controls with aspirin-tolerant asthma and controls without asthma. When compared with controls, granulocytes from subjects with AERD had comparable levels of EP₂ protein expression and PGE₂-mediated cAMP accumulation, yet were resistant to PGE₂-mediated suppression of LT generation. Percentages of platelet-adherent neutrophils correlated positively with LTB₄ generation and inversely with responsiveness to PGE₂-mediated suppression of LTB₄. The PKA inhibitor H89 potentiated LTB₄ generation by control granulocytes but was inactive in granulocytes from individuals with AERD and had no effect on platelet P-selectin induction. Both tonic PKA activity and levels of PKA catalytic gamma subunit protein were significantly lower in granulocytes from individuals with AERD relative to those from controls.

Conclusions—Impaired granulocyte PKA function in AERD may lead to dysregulated control of 5-lipoxygenase activity by PGE₂, whereas adherent platelets lead to increased production of LTs, which contributes to the features of persistent respiratory tract inflammation and LT overproduction.

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Keywords

Samter's triad; aspirin-exacerbated respiratory disease; AERD; aspirin triad; nonsteroidal anti-inflammatory drug; asthma; leukotriene; protein kinase A; prostaglandin E₂; cyclic AMP

Aspirin-exacerbated respiratory disease (AERD) is characterized by adult-onset asthma, nasal polyposis, and respiratory reactions caused by drugs that block COX-1.¹ AERD accounts for a disproportionate percentage of patients with severe asthma² and refractory nasal polyposis.³ AERD is noteworthy for intense inflammation of the respiratory mucosa, with large numbers of activated tissue eosinophils, mast cells, monocytes, and neutrophils.^{4–6} Each of these cells expresses 5-lipoxygenase (5-LO), the enzyme necessary to convert arachidonic acid into leukotrienes (LT), which are potent mediators of inflammation central to AERD. The 5-LO inhibitor zileuton substantially improves control of respiratory symptoms and diminishes the severity of reactions to aspirin,^{7,8} which confirms the importance of 5-LO products in AERD.

Two classes of LTs result from 5-LO-mediated oxidation of arachidonic acid into the unstable intermediate LTA₄: cysteinyl LTs (cysLT) and LTB₄. CysLTs form in eosinophils, monocytes, basophils, and mast cells, in which LTA₄ is conjugated to reduced glutathione by LTC₄ synthase (LTC₄S).^{9,10} The parent cysLT, LTC₄, is converted extracellularly to LTD₄ and then to the stable metabolite LTE₄. CysLTs act at G protein coupled LTE₄ receptors to induce bronchoconstriction, vascular leak, and eosinophilia.^{13–15} Persistently elevated urinary levels of LTE₄ are a hallmark of AERD.¹⁶ Neutrophils, the most numerous 5-LO-expressing cell in the blood, lack LTC₄S but hydrolyze LTA₄ to LTB₄. The urinary levels of an LTB₄ metabolite increase in parallel with LTE₄ during reactions to COX-1 inhibitors in AERD,¹⁷ which implies that the disease involves a disturbance in the control of 5-LO activity across cell types. Cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA)¹⁸ is the best characterized regulator of 5-LO activity. PKA exists as a complex of 2 catalytic subunits and 2 regulatory subunits. cAMP binds the PKA regulatory subunits, disassociating them from the catalytic subunits, which, in turn, phosphorylate intracellular substrates. Phosphorylation by PKA at serine 523 prevents 5-LO from translocating to the nuclear envelope, which precludes synthesis of LTA₄. Accordingly, stimuli that increase PKA activity, including ligands for receptors that couple to stimulatory G proteins, suppress 5-LO activity and LT generation by effector cells.¹⁹ Prostaglandin (PG) E₂ is among the most potent activators of cAMP and PKA, primarily by signaling through stimulatory G-linked E prostanoide₂ (EP₂) receptors. Both faulty PGE₂ production and impaired expression of EP₂ receptors are reported in tissues from patients with AERD.^{20–23}

In inflammation, platelets can adhere to granulocytes via P-selectin (CD62P).^{24,25} This interaction can amplify cysLT formation, as platelet-derived LTC₄S converts excess LTA₄ from granulocytes into LTC₄.²⁶ We recently reported that the percentages of platelet-adherent neutrophils, eosinophils, and monocytes in the peripheral blood and sinonasal tissues from subjects with AERD far exceeded the percentages found in controls with aspirin-tolerant asthma (ATA) and controls without asthma.²⁷ These percentages correlated with basal urinary levels of LTE₄, and adherent platelets accounted for approximately 60%

of the LTC₄S activity in granulocytes from subjects with AERD. We also found that granulocytes from individuals with AERD generated 2-fold more total 5-LO pathway products than did those from controls, which suggests an overall increase in 5-LO activity. We now demonstrate that granulocytes from individuals with AERD resist suppression of LT generation by PGE₂ and EP receptor-selective agonists, despite normal levels of EP receptor expression. Granulocytes from subjects with AERD possess lower levels of PKA activity than do granulocytes from aspirin-tolerant controls. Furthermore, the frequencies of neutrophil-adherent platelets correlate strongly with the production of LTB₄ and inversely with ability of PGE₂ and EP₂ receptors to block LTB₄ generation. Impaired granulocyte PKA function likely conspires with adherent platelets to dysregulate PGE₂-dependent 5-LO activity in AERD, both of which contribute to the pathophysiology of the disease.

METHODS

Patient characterization

Patients were recruited from the allergy, pulmonary, and otolaryngology clinics at the Brigham and Women's Hospital (Boston, Mass). Controls without asthma had no history of asthma or intolerance to nonsteroidal anti-inflammatory drugs. Controls with ATA had physician-diagnosed asthma and had taken a nonsteroidal anti-inflammatory drug within the previous 6 months without an adverse reaction. AERD was diagnosed based on asthma, nasal polyposis, a history of respiratory reaction upon ingestion of a COX inhibitor, and a confirmatory graded oral challenge to aspirin that resulted in sinonasal symptoms and/or a decrease in FEV₁ of >15%.²⁸ No subjects smoked. All subjects with AERD were treated with the CysLT₁R blocker montelukast during the aspirin challenge, and none were on zileuton. Clinical data, including pulmonary function, the presence of atopy (>2 positive skin prick tests to common inhalant allergens), and use of corticosteroids and long-acting β -agonists are summarized in Table E1 (in this article's Online Repository at www.jacionline.org). The Brigham and Women's Hospital Institutional Human Subjects Institutional Review Board approved the study, and all the subjects provided written consent.

Flow cytometry

Heparinized whole blood was assayed within 1 hour of collection. For subjects undergoing aspirin challenge, blood was collected before aspirin ingestion. Granulocytes were classified as eosinophils or neutrophils according to their scatter characteristics and expression of CD45 and CCR3. Granulocytes were assessed for the presence of adherent platelets by expression of CD61 and for the formyl peptide (fMLP) receptor 1 (all antibodies from BD Biosciences, San Jose, Calif).

EP₂ receptor expression was analyzed on permeabilized whole blood by using a mouse anti-human monoclonal antibody directed against a fusion peptide based on extracellular portions of the human EP₂ receptor (Abmart, Shanghai, China) or an IgG1 isotype control. Samples were incubated with a secondary antibody and with an antibody specific for CD45, and were fixed and analyzed as above. To confirm specificity, the EP₂ receptor antibody was tested against human embryonic kidney 293 cells (American Type Culture Collection, Manassas,

Va) transfected with human EP₂ receptors (see Fig E1 in this article's Online Repository at www.jacionline.org).

To assess platelet activation, platelet-rich plasma was incubated with or without 10 μ M H89 at 37°C for 30 minutes. Samples were incubated at room temperature for 15 minutes in the absence or presence of PGE₂ (0.01–1 μ M, Cayman Chemical, Ann Arbor, Mich), or agonists of the EP₁ receptor (DI-004), EP₂ receptor (AE1-259-01), EP₃ receptor (AE-248), or EP₄ receptor (AE-329) (Ono Pharmaceutical Co, Osaka, Japan) at 1 μ M and then stimulated for 15 minutes with 100 μ M adenosine diphosphate 5'-[β -thio] diphosphate (ADP β S) (Sigma-Aldrich, St Louis, Mo). Platelet activation was assessed by cytofluorographic analysis of CD62P (BD Biosciences). Data are expressed as a fold change from the percentage CD62P expression induced by ADP β S stimulation alone.

LT and thromboxane measurements

Aliquots of 0.5×10^6 granulocytes were primed with 700 pM granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ), 10 μ M cytochalasin-B (Sigma-Aldrich), 1.2 nM TNF- α (eBiosciences, San Diego, Calif), and 0.1 U/mL adenosine deaminase (Sigma-Aldrich) at 37°C for 30 minutes¹⁸; 10 μ M H89 or 5 μ M myristoylated 14–22 amide (Milli-pore, Billerica, Mass) was added to some samples during priming to block PKA. Some samples were pretreated with 50 μ M forskolin (Sigma-Aldrich); 0.01–1 μ M PGE₂; or 1 μ M EP₂, EP₃, or EP₄ receptor-selective agonists, and then stimulated with 1 μ M fMLP for 10 minutes. Reactions were stopped with methanol, and LTB₄, cysLTs, and thromboxane B₂ levels were measured in the supernatants by enzyme immunoassay (Cayman Chemical).

cAMP measurements

Aliquots of 100×10^6 platelets or 2×10^6 granulocytes were incubated with 200 μ M 3-isobutyl-1-methylxanthine (Sigma-Aldrich) at 37°C for 10 minutes and treated with 50 μ M forskolin, 1 pM iloprost (a selective agonist of the prostacyclin receptor) (Cayman Chemical), 0.01–1 μ M PGE₂, or 1 μ M EP receptor-selective agonists at 37°C for 5 minutes. cAMP levels were analyzed by using enzyme immunoassay (Cayman Chemical).

Western blot analysis

Western blotting of granulocyte protein was performed as described.²⁹ Membranes were probed with polyclonal antibodies against PKA catalytic α subunit (PKAC α , Cell Signaling, Danvers, Mass), PKAC β , PKAC γ , PKA regulatory I β subunit (PKARI β), PKARI α (all from Santa Cruz Biotechnology, Santa Cruz, Calif); PKARI α (Thermo Scientific, Waltham, Mass), or a monoclonal antibody against PKARI β (Santa Cruz Biotechnology). PKA subunits were quantified by densitometry and expressed relative to GAPDH.

PKA activity analysis

Basal PKA activity was measured on lysates from aliquots of 0.1×10^6 unfractionated granulocytes by using a PKA kinase activity enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY).

Statistical analysis

The data are presented as mean ($\pm$ SEM) unless otherwise stated. Differences in values were analyzed with the *t* test because all data presented were normally distributed; significance was defined as $P < .05$, and all tests were 2 tailed. Effect size was measured with the Pearson correlation coefficient.

RESULTS

Patient characteristics

The 3 groups of patients were similar in age and sex. The 2 groups of subjects with asthma did not differ in baseline FEV₁, the presence of atopy, or use of inhaled corticosteroids (Table E1). The percentage of circulating eosinophils was highest in the subjects with AERD ($10.3\% \pm 8.1\%$, $P < .05$ compared with controls without asthma and controls with ATA) (Table E1).

Increased LT generation and impaired suppression of LT production by PGE₂ and EP agonists in granulocytes from subjects with AERD

To determine whether LT generation by stimulated granulocytes differed between subjects with AERD and the controls, we measured LT levels in supernatants from primed granulocytes stimulated with $1 \mu\text{M}$ fMLP.¹⁸ Because neutrophils activated poorly after elution from an immunomagnetic CD16 selection column, all experiments were done by using unfractionated granulocytes. fMLP-stimulated granulocytes from subjects with AERD generated significantly more LTB₄ than did granulocytes from the controls without asthma ($P < .05$), with intermediate levels of LTB₄ generated by granulocytes from controls with ATA (Fig 1, A). Similar trends were seen among the patient groups for the generation of cysLTs (Fig 1, B). Cell surface expression of the fMLP receptor by both neutrophils and eosinophils did not differ significantly between the patient groups ($n = 3$ for each group, data not shown).

To determine whether EP receptor-mediated suppression of LT generation differed between granulocytes from subjects with AERD and controls, we studied the effects of pretreatment with PGE₂ and EP receptor-specific agonists. We used forskolin as a control to activate adenylyl cyclase independently of EP receptor function. Pretreatment with forskolin, PGE₂, and agonists of EP₂, EP₃, and EP₄ receptors all significantly inhibited the generation of LTB₄ by granulocytes from ATA and controls without asthma ($P < .05$ for all conditions, raw data not shown; percentage inhibition is shown in Fig 1, C). Forskolin ($P < .01$) and the EP₃ agonist ($P < .05$) also significantly suppressed LTB₄ generation by granulocytes from subjects with AERD, whereas suppression by PGE₂ ($P = .052$), the EP₂ agonist ($P = .052$), and the EP₄ agonist ($P = .07$) approached significance (raw data not shown). When the percentage suppression of LTB₄ generation was compared across groups, the effects of each agonist on the granulocytes from individuals with AERD were significantly less robust than their effects on control subjects (Fig 1, C). Trends for cysLT production were comparable with those for LTB₄, except that PGE₂ and the EP₃ and EP₄ agonists tended to potentiate cysLT generation by granulocytes from subjects with AERD (Fig 1, D). Thromboxane A₂

generation by granulocytes was not suppressed by PGE₂, forskolin, or EP receptor agonists ($n = 2$, one subject from each control group; data not shown).

Adherent platelets correlate with LTB₄ production and PGE₂-EP₂ resistance in AERD

To determine whether platelet adherence to neutrophils was related to LTB₄ generation, we examined the relationship between the platelet-adherent neutrophils in whole blood and the fMLP-induced generation of LTB₄ by granulocytes in the same samples. The percentages of platelet-adherent neutrophils in subjects with AERD correlated strongly with the basal fMLP-stimulated LTB₄ generation ($r = 0.89$) (Fig 2, *A*) and correlated negatively with the percentage suppression of LTB₄ release by pretreatment with either PGE₂ ($r = -0.65$) or the EP₂ agonist ($r = -0.71$) (Fig 2, *B* and *C*). Correlations were also found in control groups (percentages of platelet-adherent neutrophils vs percentage suppression of LTB₄ by PGE₂ in controls without asthma and controls with ATA was $r = -0.27$ and -0.48 , respectively, and vs percentage suppression of LTB₄ by EP₂ was $r = 20.64$ and 20.76 , respectively, not shown).

EP₂ receptor protein expression and EP receptor-induced cAMP accumulation are not impaired in AERD

Neutrophils consistently expressed EP₂ receptor protein (Fig 3, *A* and *B*). No differences in EP₂ expression were noted among the 3 patient groups. Eosinophils (Fig 3, *B*, bottom) and platelets (Fig 3, *D*) in the same samples expressed lower EP₂ levels than neutrophils, with no differences among the patient groups. Granulocytes (Fig 3, *C*) and platelets (Fig 3, *E*) from all 3 groups of patients accumulated cAMP in response to forskolin, PGE₂, and agonists of the EP₂ and EP₄ receptors, with no differences between subjects with AERD and control subjects. Platelets in the 3 groups responded equivalently to iloprost (Fig 3, *E*). Neither the EP₁ nor the EP₃ receptor-selective agonist induced cAMP accumulation in the cells of any patient group. Platelets from all 3 subject groups expressed CD62P in response to ADPβs stimulation (Fig 3, *F*), with no differences in the magnitude of response. Whereas 1 μM PGE₂ tended to potentiate CD62P induction, EP₂ and EP₄ agonists tended to suppress, as did forskolin and iloprost. Again, there were no differences among the patient groups.

PKA inhibition fails to potentiate LTB₄ production in AERD

To determine whether control of 5-LO activity by PKA differed among the subject groups, fMLP-induced LTB₄ production was measured in the absence or presence of H89. In some experiments we compared the effect of H89 with that of a myristoylated peptide PKA inhibitor (myrPKI, 5 μM) to verify specificity. We activated cells with and without PGE₂-EP agonist pretreatment to assess both the effect of inhibiting tonic PKA activity and the effect of PKA inhibition on EP receptor-mediated suppression of LTB₄ generation. In the absence of pretreatment, PKA inhibition significantly potentiated LTB₄ generation by granulocytes from both control subjects without asthma and those with ATA (Fig 4, *A* and *B*). Similar trends were observed for cysLT generation (data not shown). In 3 healthy controls, the effect of myrPKI on fMLP-induced LTB₄ generation was similar to that of H89 (from 259 ± 23 pg LTB₄/mL to 455 ± 140 pg LTB₄/mL with H89, and 378 ± 76 pg LTB₄/mL with myrPKI, respectively). H89 also significantly reversed the suppression of

LTB₄ generation caused by pretreatment with PGE₂ and the EP₂ receptor agonist in cells from controls, with similar trends for cells pretreated with EP₃ and EP₄ agonists. In contrast, H89 did not potentiate the generation of LTB₄ by granulocytes from subjects with AERD under any condition (Fig 4, C). In addition, the effect of myrPKI on fMLP-induced LTB₄ generation also was similar to that of H89 in 3 subjects with AERD (4271 ± 1698 pg LTB₄/mL to 4962 ± 1193 pg LTB₄/mL with H89 and 4751 pg LTB₄/mL $\pm$ 1724 pg LTB₄/mL with myrPKI, respectively). Treatment of platelets with H89 did not alter the expression of CD62P in response to ADP β S and did not reverse the effects of EP agonists, iloprost, or forskolin in any patient group (data not shown).

Granulocyte PKA activity and PKAC γ subunit expression are reduced in AERD

Freshly isolated granulocytes were analyzed for PKA activity and PKA protein. Baseline granulocyte PKA activity, measured by using enzyme immunoassay, was lower in subjects with AERD than in the controls without asthma (Fig 5, A). The levels of granulocyte PKA activity correlated significantly with the extent of potentiation of LTB₄ by treatment with H89 ($r = 0.49$, $P = .05$) (Fig 5, B) but not with the total amount of LTB₄ generated (data not shown). Granulocytes from all controls without asthma and control subjects with ATA contained PKAC γ protein (representative Western blot) (Fig 5, C), with considerable intersubject variability. Corrected PKAC γ protein levels were significantly lower in subjects with AERD (Fig 5, D) than in controls. Granulocyte PKAC α and PKAC β proteins were low to undetectable in all groups (data not shown). PKARI α and PKARI β subunits were detected in all samples and their levels did not differ among the groups (data not shown). PKARI α or PKARI β subunits were not detected. Lysates from washed platelets strongly expressed all 3 PKA catalytic subunits as well as PKARI α and PKARI β subunits (data not shown), which verified the efficacy of the antibodies. Removal of platelets by trypsinization did not alter levels of PKA subunits or PKA activity.

DISCUSSION

Persistent respiratory tract inflammation and overproduction of LTs are hallmarks of AERD. The 5-LO products are prominent disease effectors,⁷ especially during the reactions that occur with administration of nonselective COX inhibitors.^{8,30} Such reactions are characterized by the marked further overproduction of both cysLTs¹⁶ and LTB₄.¹⁷ Although these findings suggest that AERD involves a fundamental disturbance in the control of 5-LO, no explanatory mechanism had previously been demonstrated. PGE₂, an abundant COX product, can suppress 5-LO activity by inducing PKA signaling through EP₂ (and possibly other) receptors,^{18,19} and analysis of results of immunohistochemical studies suggest that EP₂ receptor protein expression was reduced on several leukocyte subsets in the sinonasal²¹ and bronchial mucosa²⁰ from patients with AERD relative to controls with ATA. However, no previous study had examined whether EP receptor-mediated control of 5-LO activity was impaired in cells from patients with AERD.

We activated granulocytes by using fMLP, a physiologic agonist that acts through the formyl peptide G protein coupled receptors expressed by both neutrophils³¹ and eosinophils.³² Under priming conditions to amplify the fMLP signal,¹⁸ granulocytes from all

3 subject groups generated both classes of LTs, with higher levels generated by cells from subjects with AERD (Fig 1, A and B). This difference was not attributable to differences in fMLP receptor expression and is also consistent with our prior study in which levels of total 5-LO products generated by ionophore-stimulated granulocytes from subjects with AERD were approximately 2-fold higher than levels generated by granulocytes from controls with ATA.²⁷ CysLTs can be generated both by eosinophils and by platelet-adherent neutrophils. In our current study, the generation of cysLTs correlated modestly with both the percentages of eosinophils ($r = 0.65$) and platelet-adherent granulocytes ($r = 0.58$) in the samples, which supports the thesis that 5-LO activity may be increased across cell types in AERD.

As expected, PGE₂ dose dependently suppressed fMLP-induced LTB₄ generation in cells from control subjects. However, suppression in the cells from subjects with AERD was markedly blunted (Fig 1, C). Similar trends were observed for cysLTs, although the EP₄ agonist and, to a lesser extent, EP₃ agonist tended to potentiate cysLT formation in the granulocytes from subjects with AERD (Fig 1, D). The potentiation of cysLTs may be due to different effects of EP receptor signaling in eosinophils (more abundant with subjects with AERD than with controls) versus neutrophils. We anticipated that EP₂ receptor signaling would dominate in its ability to block LTB₄ formation and that its function would be impaired in AERD. Indeed, the percentage suppression of LTB₄ generation by pretreatment with PGE₂ and the EP₂ agonist correlated significantly ($r = 0.73$, $P = .01$), which suggests that the effects of PGE₂ reflected, at least in part, signaling through EP₂.

Three unexpected findings emerged from these studies. First, LTB₄ production in controls was suppressed not only by the EP₂ receptor agonist but also by agonists for the EP₃ and EP₄ receptors at doses at which they are specific for their targets.³³ The administration of the EP₃ agonist suppressed LT generation in allergen challenged mouse lung³⁴ and also blocked cysLT release in response to aspirin challenge in a mouse model of AERD.^{35,36} Collectively, these studies and the current result support the capacity of EP₃ receptor signaling to suppress LT generation *in vitro* and *in vivo*, despite the inability of the EP₃ agonist to raise cAMP levels. None of the agonists suppressed thromboxane A₂ formation in the same samples, which supports the conclusion that the agonists targeted 5-LO activity rather than arachidonic acid release. Second, the impaired response of the granulocytes from subjects with AERD extended to all of the agonists, including forskolin (Fig 1, C and D). Third, the quantity of LTB₄ generated correlated positively with the frequencies of adherent platelets (Fig 2, A), whereas the extent to which PGE₂ and the EP₂ agonist suppressed LTB₄ formation correlated negatively with platelet adherence (Fig 2, B and C). In a mouse model of AERD, antibody-mediated platelet depletion markedly suppressed the production of both LTB₄ and cysLTs in response to aspirin challenge, which suggests that platelet adherence contributes substantially to the dysregulation of 5-LO activity.³⁵ We, therefore, focused on whether AERD involved a signaling defect downstream of EP receptors, and, if so, whether the defect involved granulocytes, platelets, or both.

Results of previous immunohistochemical studies suggested that expression of the EP₂ receptor protein is specifically reduced on effector cells in bronchial and nasal biopsy specimens from subjects with AERD compared with controls who are aspirin tolerant.^{20,21} In contrast, we found no differences in EP₂ receptor protein expression by neutrophils (Fig

3, B, top), eosinophils (Fig 3, B, bottom), or platelets in peripheral blood from subjects with AERD relative to controls, nor did we find evidence for defective EP₂ receptor function (Fig 3, C and D). EP₂ receptor expression is upregulated on sputum eosinophils compared with peripheral blood eosinophils³⁷ and can be either upregulated²² or downregulated³⁸ by cytokines or ligands for Toll-like receptors. It therefore is possible that the expression of EP₂ on leukocytes is modified differently in the tissues of subjects with AERD than in controls once they are recruited. Moreover, responses to the EP₄ agonist, forskolin, and, in the case of platelets, iloprost indicated that the associated stimulatory G proteins and adenylyl cyclases were functionally intact. Platelet adhesion onto granulocytes requires expression of platelet CD62P, and, because CD62P expression is controlled by cAMP, we studied CD62P induction as a surrogate for downstream signaling of platelet EP receptors. Platelets from subjects with AERD were not different from those of controls in terms of activation-induced CD62P expression, and suppression of this expression by EP₂ and EP₄ agonists was unimpaired (Fig 3, F). Thus, we turned our focus to PKA as the next logical post-receptor-signaling step to explain the relatively PGE₂-resistant phenotype exhibited by granulocytes from subjects with AERD.

PKA-mediated phosphorylation of 5-LO controls both its catalytic activity³⁹ and its nuclear import.⁴⁰ We used H89, a PKA inhibitor, to determine whether control of 5-LO function by PKA activity was impaired in granulocytes from subjects with AERD. H89 doubled the production of LTB₄ by fMLP-stimulated granulocytes from controls without asthma and controls with ATA. Responses to myrPKA were similar to responses to H89 in all groups. H89 also completely reversed the PGE₂- and EP₂-mediated suppression of LTB₄ (Fig 4, A and B), while also potentiating LTB₄ generation in the presence of EP₃ and EP₄ agonists. Because the stimulation with EP₃ receptor agonist did not result in increased cAMP, the reversal of its effect on LTB₄ production could reflect either an off-target effect of H89 or could reflect an alternative pathway to PKA activation as reported elsewhere.⁴¹ Nevertheless, the cells from subjects with AERD resisted the effects of H89 (Fig 4, C), which suggests that a disturbance of PKA function may impair the ability of PGE₂ to suppress granulocyte 5-LO in AERD. Moreover, the finding that H89 did not alter the ADPβS-induced expression of platelet CD62P in any subjects suggested that the functional disturbance in PKA in AERD rested in granulocytes.

Although basal PKA activity varied substantially, it was significantly lower in granulocytes from subjects with AERD (Fig 5, A), including the individuals with the 4 lowest values. PKA activity correlated significantly with the potentiation of LTB₄ generation in response to H89 (Fig 5, B). PKAC γ was the catalytic subunit expressed most consistently and strongly in granulocytes (Fig 5, C), which contained negligible amounts of PKAC α and β subunits. PKAC γ was expressed at lower levels in granulocytes from subjects with AERD when corrected for GAPDH (Fig 5, D), including the 4 individuals with the lowest amounts among all analyzed. The small numbers of individuals for whom both PKA activity and PKAC γ data were available (n = 8 [2 with AERD]) did not permit an analysis of correlation. PKAC γ levels are among several variables, including basal cAMP levels, the cell activation state *in vivo*, *in vivo* exposure to PGE₂, the strength of association of catalytic and regulatory subunits, and artifacts of handling that could influence PKA activity. It is noteworthy that

the expressions of EP₂ receptors and PKA α subunits are variably deficient in lung fibroblasts from patients with idiopathic pulmonary fibrosis.⁴² Both defects result in relative resistance to the growth-suppressive effects of PGE₂. Regardless of cause, reduced PKA function in granulocytes may have several effects not addressed by our study. These could include failure to induce EP₂ expression in the inflamed tissues because the human *PTGER2* gene contains consensus binding sites for PKA-dependent transcription factors.

Although blood granulocytes may contribute to the basal production of LTs in AERD,²⁷ resident tissue cells such as mast cells may contribute substantially to the sharp rise of LTs that occur in response to aspirin challenges. Inhaled PGE₂ blocks both bronchoconstriction and increases in urinary LTE₄ that occur in response to aspirin challenge.⁴³ These bronchoprotective effects of exogenous PGE₂ may reflect actions on mast cells or other resident tissue cells that retain PGE₂ responsiveness. Indeed, mast cells cultured from peripheral blood progenitors of patients with AERD exhibit robust suppression of IgE-dependent cysLT production in response to preincubation with PGE₂.⁴⁴ It also is possible that the doses used in the *in vivo* study (70 μ M) achieved local concentrations high enough to overcome any deficit in either EP receptor or PKA function. PGE₂ normally increases with pulmonary inflammation and limits maximal 5-LO activation *in vivo*.³⁵ The relative resistance of cells from subjects with AERD to PGE₂ identified in our study could compound their capacity for high-level LT production (Fig 1) and promoted LT-related respiratory symptoms at baseline. These symptoms become marked when local PGE₂ levels fall below a critical threshold when COX-1 is inhibited by aspirin ingestion. To our knowledge, this is the first study linking platelets to 5-LO function in humans and demonstrating a functional impairment of the PKA system in granulocytes from carefully phenotyped subjects with AERD. An *in vivo* dose-ranging study would be necessary to assess whether patients with AERD are relatively hyporesponsive to inhaled PGE₂.

Platelet adhesion onto granulocytes increases granulocyte integrin avidity and expression⁴⁵ and permits transcellular synthesis of cysLTs.⁴⁶ The strong correlation between the percentages of neutrophils with adherent platelets *in vivo* and the absolute levels of LTB₄ generated by fMLP-stimulated granulocytes *in vitro* (Fig 2, A) in this study may reflect priming of 5-LO by soluble or contact-dependent factors from platelets.⁴⁷ However, platelet adherence also correlated negatively with the extent of suppression of LTB₄ generation by pretreatment with PGE₂ and the EP₂ receptor agonist (Fig 3, B and C). Interactions between granulocytes and platelets are multifactorial and bilateral, and we cannot exclude the possibility that platelets actively suppress PKA activity in granulocytes. It also is possible that impaired PKA activity dysregulates the release of factors from neutrophils, which, in turn, activate platelet expression of CD62P.⁴⁸ Tonic PKA activity in granulocytes also controls integrin expression and avidity,⁴⁹ a parameter that correlates with adherence to platelets in AERD.³⁴ Thus, impaired PKA activity, by simultaneously facilitating adherence of platelets, enhancing integrin expression, and impairing cAMP-mediated control of 5-LO activation, would promote the severe, persistent respiratory tract inflammation and LT overproduction that are characteristic of AERD (see Fig E2 in this article's Online Repository at www.jacionline.org).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

5-LO	5-lipoxygenase
ADPβS	Adenosine diphosphate 5'-[β-thio]diphosphate
AERD	Aspirin-exacerbated respiratory disease
ATA	Aspirin-tolerant asthma
cAMP	Cyclic adenosine monophosphate
cysLT	Cysteinyl leukotriene
EP	E prostanoid receptor
fMLP	Formyl peptide
LT	Leukotriene
LTC₄S	Leukotriene C ₄ synthase
myrPKI	Myristoylated peptide protein kinase A inhibitor
PG	Prostaglandin
PKA	Protein kinase A

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FIG 1. LT production by fMLP-stimulated granulocytes and suppression by PGE₂ and EP agonists. LTB₄ (**A**) and cysLT (**B**) production by 0.5×10^6 granulocytes stimulated with 1 μM fMLP are shown from controls without asthma (*white columns*), controls with ATA (*gray columns*), and subjects with AERD (*black columns*). Percentage suppression of LTB₄ (**C**) and cysLT (**D**) production by fMLP-stimulated granulocytes pretreated with the listed agonists is shown for cells from controls without asthma, controls with ATA, and subjects with AERD. Data are expressed as mean ($\pm$ SEM) (* $P < .05$; ** $P < .01$).

FIG 2.

Platelet-adherent neutrophils, LTB₄ generation, and suppression of 5-LO activity in AERD. **A**, Percentages of platelet-adherent neutrophils in whole blood from 8 subjects with AERD are plotted against quantity of LTB₄ generated by fMLP-stimulated granulocytes from the same individuals. Percentage suppression of fMLP-induced LTB₄ by pretreatment with PGE₂ (**B**) or the EP₂ receptor-specific agonist (**C**) plotted against percentages of platelet-adherent neutrophils in the blood of each subject. Effect size (Pearson correlation) is denoted as an r value.

FIG 3.

EP₂ protein expression, cAMP accumulation, and inhibition of platelet CD62P. **A**, Representative flow cytometric histogram of intracellular EP₂ is shown for neutrophils from a subject with AERD. Mean intra-cellular EP₂ levels of neutrophils and eosinophils (**B**) and of platelets (**D**) are shown for controls without asthma (n = 6), controls with ATA (n = 5), and subjects with AERD (n = 16). Intracellular cAMP accumulation within granulocytes (**C**) or platelets (**E**) stimulated with listed agonists is shown. **F**, Inhibition of ADPβS-stimulated CD62P expression on platelets in platelet-rich plasma is shown. Data are expressed as mean (±SEM). There were no significant differences between patient groups for any comparisons in panels B–F.

FIG 4.

Effect of H89 on LTB₄ production from blood granulocytes. The effect of pretreatment of granulocytes with H89 on fMLP-induced LTB₄ production is shown for controls without asthma (**A**) (n = 6), controls with ATA (**B**) (n = 6), and subjects with AERD (**C**) (n = 8). Data are expressed as mean ($\pm$ SEM) (* P < .05; ** P < .01).

FIG 5.

Granulocyte PKA activity and PKAC γ subunit expression. **A**, Basal PKA activity measured by ELISA is shown. **B**, The net effect of H89 on granulocyte production of LTB₄ is shown plotted against the basal PKA activity for each patient. Effect size is denoted as an r value. **C**, Representative Western blot of granulocytes for PKAC γ protein, with GAPDH shown below. Of note, the 2 control subjects with ATA with the lowest PKAC γ protein levels of that patient group are the 2 shown on this blot. **D**, Mean PKAC γ protein levels normalized to GAPDH are shown. * $P < .05$.