Nonsteroidal anti-inflammatory–induced inhibition of signal transducer and activator of transcription 6 (STAT-6) phosphorylation in aspirin-exacerbated respiratory disease

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Background: Aspirin desensitization provides long-term clinical benefits. The exact mechanisms of aspirin desensitization are not clearly understood.

Objective: We sought to evaluate the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on T-cell activation of the IL-4 pathway in aspirin-sensitive patients with asthma and control subjects.

Methods: A total of 11 aspirin-sensitive patients with asthma, 10 aspirin-tolerant patients with asthma, and 10 controls without asthma were studied. PBMCs were stimulated with an anti-CD3 antibody and IL-4 or IL-12, with and without the presence of NSAIDs. The expression of phosphorylated signal transducers and activators of transcription 6 (pSTAT6), phosphorylated signal transducers and activators of transcription 4, and IL-4 was detected in CD4 T cells by flow cytometry.

Results: Stimulation with a combination of anti-CD3 and IL-4 induced pSTAT6 in CD4 T cells from all subjects. The induction of pSTAT6 was significantly higher in aspirin-sensitive patients with asthma than in controls subjects. The increase in pSTAT6 was inhibited in a dose-dependent manner by aspirin and indomethacin and minimally by sodium salicylate. This inhibition was strongest in aspirin-sensitive patients. Two-group comparisons showed significant differences in pSTAT6 inhibition by all concentrations of indomethacin and aspirin: between aspirin-sensitive and aspirin-tolerant groups and between aspirin-sensitive and control groups. No differences were found between aspirin-tolerant and control groups at all 3 concentrations. The inhibition of pSTAT6 was associated with reduced IL-4 expression.

Conclusions: NSAIDs inhibited signal transducers and activators of transcription 6 signaling in CD4 T cells. This inhibition was significantly higher in aspirin-sensitive patients than in aspirin-tolerant subjects and was associated with reduced expression of IL-4. These findings have implications for clinical benefits of aspirin desensitization in aspirin-sensitive patients with asthma. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Aspirin-exacerbated respiratory disease, IL-4 signaling inhibition, pSTAT6 inhibition, mechanism of aspirin desensitization*

It has been almost 100 years since Widal et al[1] first described aspirin challenges and desensitization for patients with the syndrome of asthma, nasal polyposis, and aspirin intolerance. Later, following works by Samter and Beers,[2] the condition was termed Samter's triad because of the association of these 3 overlapping conditions. Subsequent studies have shown that aspirin desensitization therapy provides significant clinical improvement in nasal symptoms and a reduction in corticosteroid use.[3] Symptom improvement has been reported to occur as early as 4 weeks after the initiation of aspirin therapy, and long-term benefits include significant reductions in the number of sinus infections and operations, hospitalizations for asthma, and use of systemic steroids.[4,5] Despite these data supporting the clinical benefit, the exact mechanisms of aspirin desensitization are still not clearly understood.

Aspirin-exacerbated respiratory disease (AERD) is characterized by a high level of tissue, as well as blood eosinophils, suggesting a type 2 immune response (including a T_H2 response) disorder. Recently, Steinke et al[6] reported an increased expression of IFN-γ mRNA transcripts in the nasal polyp tissue derived from subjects with AERD, as compared with aspirin-tolerant patients and control subjects. Eosinophils were the source of IFN-γ. The AERD tissue expressed high levels of not only IFN-γ but also IL-4. Interestingly, the level of IL-5 was not elevated. The noted findings primarily reflected the cytokine milieu derived from tissue eosinophils.[6] The simultaneous presence of IL-4 and IFN-γ in nasal polyps from subjects with AERD is similar to that observed in atopic dermatitis, another T_H2 disease with a mixed T_H1/T_H2 cytokine milieu. Thus, AERD may represent a mixed T_H1/T_H2 immune response disease.

AERD is associated with increased cysteinyl-leukotriene production. Urinary leukotriene E4 (LTE_4) levels correlate with the severity of aspirin response.[7] During desensitization, urine LTE_4 levels initially increase following aspirin ingestion but subsequently decrease to basal levels with continued treatment. Cysteinyl-leukotriene receptor 1 numbers also decrease following desensitization.[8-11] Recently, there has been progress in further elucidating immunologic differences in patients with AERD and differential responses following aspirin desensitization. We have shown that aspirin desensitization was associated with

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Abbreviations used
AERD: Aspirin-exacerbated respiratory disease
LTE_4: Leukotriene E4
MFI: Mean fluorescence intensity
NSAID: Nonsteroidal anti-inflammatory drug
pSTAT6: Phosphorylated signal transducers and activators of transcription 6
STAT4: Signal transducers and activators of transcription 4
STAT6: Signal transducers and activators of transcription 6
Jak1: Janus kinase 1

suppression of IL-4 and matrix metalloproteinase-9.[12] We conducted the following study to examine the mechanisms underlying the mechanism of reduced IL-4 production following aspirin desensitization. We evaluated the *in vitro* effects of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) on peripheral blood T-cell activation in aspirin-sensitive and aspirin-tolerant patients with asthma, as well as in healthy controls.

METHODS

Subjects

Subjects were recruited from our outpatient clinic at National Jewish Health in Denver, Colo. Subjects were selected on the basis of their history of AERD and included a diagnosis of asthma, chronic hyperplastic sinus disease with nasal polyposis, and a convincing history of aspirin sensitivity. Patients with AERD had already agreed to undergo aspirin challenge and desensitization in consultation with their primary physician before recruitment. Asthma was based on physician diagnosis. All subjects had a previous positive methacholine and historical reversibility of at least 12% and 200 cc, respectively, and were not on oral steroids. A total of 11 aspirin-sensitive patients with asthma underwent aspirin challenge and desensitization as described previously, although in this experiment we studied baseline data as described below.[12] We also studied 10 aspirin-tolerant patients with asthma as well as 10 controls without asthma. Controls without asthma included 6 subjects with allergic rhinitis without asthma and 4 healthy subjects.

Blood collection

Protocol for serum blood draw was approved through the Institutional Review Board of National Jewish Health (HS no. 2591). Approximately 50 mL of blood was collected in EDTA anticoagulated (lavender top) tubes from each subject on completion of appropriate consent. Blood was collected before aspirin challenge and was kept at room temperature until processing within 1 to 3 hours of collection.

Aspirin inhibition and IL-4 stimulation

PBMCs were isolated by using Ficoll-Hypaque (Histopaque-1077; Sigma-Aldrich, St Louis, Mo) gradient centrifugation. Cell culture plates were coated with 2 μg/mL of antihuman CD3 antibody (clone OKT3; eBioscience, San Diego, Calif). PBMCs were cultured in the coated plates in RPMI 1640 with 10% FBS. NSAID stock solutions of 30 mM, 100 mM, and 300 mM were made by dissolving aspirin (acetylsalicylic acid), indomethacin, and sodium salicylate (Sigma-Aldrich) in ethanol as a vehicle. Following overnight culture, equal volumes of the appropriate NSAID stock solution, or ethanol, were added to PBMC cell cultures for final concentrations of 0.3 mM, 1 mM, and 3 mM. This concentration (1%) of ethanol did not affect cell viability, signal transducers and activators of transcription 6 (STAT6) phosphorylation, or IL-4 production in our experiments. After an additional 24 hours, PBMCs were stimulated with 20 ng/mL of human recombinant IL-4 for 20 minutes. In select experiments, IL-12, instead of IL-4, was used. For measurement of intracellular IL-4, the culture with IL-4 was extended for 3 days. One sample of PBMCs from these cultures was then washed extensively, fixed with 4% paraformaldehyde solution for 15 minutes, and processed for staining and flow cytometry as described below. In another set of experiments, PBMCs were cultured as above for 2 days after the addition of IL-4. RNA was isolated and used for measurement of mRNA for IL4 by real-time PCR as described previously.[13] The primers for IL-4 were as follows: forward, ACTTTGAACAGCCTCACA GAG; reverse, TTGGAGGCAGCAAAGATGTC; beta actin forward, AGAGC TACGAGCTGCCTGAC, reverse, AGCACTGTGTTGGCGTACAG.

Intracellular staining and analysis by flow cytometry

Fixed PBMCs were permeabilized by adding 90% methanol on ice for 30 minutes. Cells were then stained with fluorescein isothiocyanate–labeled anti–phospho-STAT6 (anti– phosphorylated signal transducers and activators of transcription 6 [pSTAT6]) antibody (clone 18), phycoerythrin-labeled anti-pSTAT4 (clone 38) (both from BD Biosciences, San Jose, Calif), phycoerythrin/Cy7-labeled anti–IL-4, and antigen-presenting cell–labeled anti-CD4 antibody (clone RPA-T4) (both from BioLegend, San Diego, Calif). Samples were then analyzed by flow cytometry (CyAn ADP analyzer; Beckman Coulter, Brea, Calif). Data analysis was completed using the FlowJo software (v 7.6.5; Tree Star, Inc, Ashland, Ore) as described previously.[13,14]

Statistical analyses

Inhibition as measured by STAT6 phosphorylation was modeled using linear mixed models. Percent inhibition was log transformed before analysis. Predictors of interest were group (aspirin-sensitive, aspirin-tolerant, and healthy controls), concentration (0.3, 1, and 3 mM), and the interaction between group and concentration. The interaction term was kept in all models to allow for model flexibility. All models contained a random intercept for each subject. Analyses were completed using Statistical Analysis System version 9.4. Additional statistical analyses for differences between groups were performed using the software GraphPad Prism 6 (San Diego, Calif). Nonparametric tests were used for all analyses.

RESULTS

We isolated PBMCs, prestimulated overnight with an anti-CD3 antibody and then incubated with 2 strong COX inhibitors (aspirin and indomethacin) and a weak COX inhibitor (sodium salicylate) for 24 hours. After this period, the cells were activated with IL-4 for 20 minutes and then assayed for phosphorylation of various signaling molecules by flow cytometry. Fig 1 shows representative flow cytograms for pSTAT6 and pSTAT4 in CD4 T cells and non-CD4 PBMCs. There was very little pSTAT6 in nonstimulated cells under basal conditions (Fig 1, *A*). Stimulation with a combination of anti-CD3 and IL-4 induced pSTAT6, which was reflected by an increase in the percentage of pSTAT6+ CD4 T cells, as well as by the increase in mean fluorescence intensity (MFI) (Fig 1, *C*). This increase occurred in both CD4 T cells and non-CD4 PBMCs. For this article, we analyzed pSTAT6 data for the CD4 T-cell population only. The increase in pSTAT6 (both percentage of positive cells and their MFI) was inhibited in a dose-dependent manner by aspirin (Fig 1, *D-F*). Isotype antibody controls are shown in Fig 1, *G*. The effect of aspirin was not specific for STAT6 because it also inhibited anti-CD3/IL12-induced phosphorylation of signal transducers and activators of transcription 4 (STAT4) (Fig 1, *H* and *I*). Using this approach, we studied inhibition, by 2 potent COX-1 inhibitors (aspirin and indomethacin) and 1 weak inhibitor (sodium salicylate), of signaling through the IL-4 pathway in 11 aspirin-sensitive, 10 aspirin-tolerant, and 10 control subjects.