

An inverse relationship between peroxisome proliferator-activated receptor γ and allergic airway inflammation in an allergen challenge model

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Background: Peroxisome proliferator-activated receptor γ (PPAR- γ) expression has not been evaluated in bronchoalveolar lavage (BAL) cells from allergic asthmatic patients.

Objective: To determine whether inappropriate down-regulation of PPAR- γ in alveolar macrophages may contribute to persistent airway inflammation in allergic asthma.

Methods: We used segmental allergen challenge as a model of in vivo experimental allergic asthmatic exacerbation and airway inflammation. PPAR- γ gene expression was evaluated at baseline and 24 hours later in asthmatic patients and controls using real-time polymerase chain reaction. Immunofluorescence was used to determine cellular location of the PPAR- γ protein.

Results: We demonstrate for the first time to our knowledge that PPAR- γ messenger RNA and protein, which are highly expressed in alveolar macrophages of healthy individuals, are significantly reduced in asthmatic patients after segmental allergen challenge. In allergic asthmatic patients ($n = 9$), PPAR- γ gene expression decreased significantly from baseline to postchallenge BAL (median decrease, 45%; $P = .008$). Furthermore, immunofluorescence staining demonstrated that PPAR- γ protein was associated with alveolar macrophages and not with inflammatory eosinophils and neutrophils.

Conclusion: Results implicate down-regulation of PPAR- γ in BAL cells as a potential factor in dysregulation of lung homeostasis in asthmatic patients. The present findings suggest that PPAR- γ agonists could have a future role in asthma therapy and warrant further study.

Ann Allergy Asthma Immunol. 2005;95:468–473.

INTRODUCTION

Asthma is characterized by chronic inflammation punctuated by exacerbations with acute inflammatory episodes that are associated with airway structural changes.¹ Although the cause(s) of the pathophysiologic features of asthma are undefined, a variety of cells and their products have been implicated, including airway epithelium, smooth muscle cells, fibroblasts, alveolar macrophages, eosinophils, and T_H2 lymphocytes.^{1,2} A wide array of mediators (eg, proinflammatory cytokines, chemokines, and metalloproteinases [MMPs]) produced by these cells have been targeted as prominent players in the different stages of asthma, leading to permanent airway alterations. Peroxisome proliferator-activated re-

ceptors (PPARs) are ligand-activated nuclear transcription factors that have been implicated in lipid metabolism and immune homeostasis.³ PPAR expression has been demonstrated in many cell types, including adipocytes, macrophages, and B and T cells.⁴ Three PPAR isoforms (α , δ (β), γ) have been identified to date, and their ligands include naturally occurring fatty acids and eicosanoids.

Suggestive evidence of therapeutic activity of synthetic PPAR- γ ligands, such as the thiazolidinediones, is accumulating in numerous diseases associated with excess cytokine and chemokine production.⁵ For example, in atherosclerotic plaques, the pathologic expression of MMPs, particularly MMP-9, which is regulated by PPAR- γ ,⁶ is thought to contribute to their instability and increase the propensity to rupture. In diabetic patients treated with rosiglitazone, a significant reduction in MMP-9 and tumor necrosis factor α in serum was noted.⁷ Ulcerative colitis, another disease accompanied by an increased production of proinflammatory cytokines, also has been associated with some improvement after rosiglitazone treatment.⁸

PPAR- γ has been noted to be constitutively present in asthmatic and healthy human airway cells, including epithe-

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This work was funded by Wyeth and the generous support of Regina Taussig.

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Received for publication November 10, 2004.

Accepted for publication in revised form February 15, 2005.

lial cells,^{9–11} alveolar macrophages,^{9,12,13} and smooth muscle cells.¹⁴ In the macrophage, PPAR- γ is thought to negatively regulate inflammatory cytokine expression^{6,15} through its ability to bind transcription factors such as NF- κ B, AP-1, and STAT-1 in a ligand-dependent manner. This phenomenon is referred to as transrepression and does not require DNA binding by PPAR- γ .^{4,16} Importantly, several *in vivo* murine ovalbumin challenge models of asthma indicate that PPAR- γ activation by exogenous ligands inhibit airway hyperactivity, influx of T_H2 cytokines and eosinophils, and structural changes to the airway wall.^{17–20} To date, PPAR- γ expression has not been evaluated in bronchoalveolar lavage (BAL) cells from allergic asthmatic patients. Based on these observations, we hypothesized that inappropriate down-regulation of PPAR- γ in BAL cells may contribute to persistent airway inflammation in allergic asthma.

To test our hypothesis, we used segmental allergen challenge in allergic asthmatic patients as an *in vivo* controlled experimental model of asthma exacerbations. We investigated PPAR- γ gene and PPAR- γ protein expression in BAL cells from allergic asthmatic patients compared with healthy volunteers both at baseline and 24 hours after a segmental allergen challenge.

MATERIALS AND METHODS

Study Population

This protocol was approved by the institutional review board, and written informed consent was obtained from all participants. Six healthy controls and 9 mild asthmatic patients were recruited for the study. Potential asthmatic patients were accepted into the study based on previously defined criteria,²¹ which briefly included the following: (1) current asthma as defined by American Thoracic Society criteria; (2) no smoking within 1 year and a total of less than 5 pack-years of smoking; (3) age between 18 and 55 years; (4) no inhaled or systemic steroids for more than 4 weeks; (5) no asthma exacerbations in the past month; and (6) allergic as defined by positive skin test results to at least 2 of a standard battery of 10 aeroallergens, one of which had to be ragweed or timothy grass.

Control subjects were enrolled if they fulfilled the following criteria: (1) absence of any cardiopulmonary symptoms; (2) no smoking within 1 year and a less than 5 pack-year history of smoking; (3) negative methacholine challenge result (per the method of Chai et al²²); (4) negative skin test results to the aeroallergen panel; and (5) age between 18 and 55 years.

Study Design

After enrollment each participant underwent a physical examination, complete history, and spirometry. Participants then underwent whole lung allergen challenge with ragweed or timothy grass (Greer Laboratories, Lenoir, NC). The allergen was chosen on the basis of skin test results for the allergic patients. All patients were challenged during a season where the allergen of interest was not in the ambient air.

Whole lung allergen challenge was performed with progressively increasing doses of allergen to determine an allergen-induced provocative dose that would cause a 20% decrease in forced expiratory volume in 1 second (FEV₁). The maximum allergen dose used was 100 PNU/mL in 1 participants, 316 PNU/mL in 4, and 1,000 PNU/mL in 4. The allergens used for whole lung allergen challenge were ragweed (6 participants) and timothy grass (3 participants). Since our study focused on allergic asthma, we did not attempt to separate asthma from atopy. The control group for nonspecific responses was composed of nonatopic, nonasthmatic subjects who received the highest dose of allergen. None of the control subjects experienced a significant decrease in their FEV₁. A minimum of 4 weeks after the aerosolized challenge, subjects underwent fiberoptic bronchoscopy under local anesthesia. A baseline BAL sample was obtained from the lingula. Saline warmed to 37°C was instilled in 50-mL aliquots (a total of 150 mL per segment) and withdrawn by gentle aspiration. Allergen was then instilled into 2 lung segments in the right middle lobe via the bronchoscope. The dose used for bronchoscopic challenge (segmental allergen challenge) was 10% of the maximum dose used in the whole lung allergen challenge diluted in 10 mL of saline solution into each of 2 segments. After the bronchoscopy, participants were observed for 2 hours and then discharged with a peak expiratory flow diary. Participants returned 24 hours later for a second bronchoscopy at which time a BAL sample was obtained from the allergen-challenged lung segments. The participants without positive skin test results were arbitrarily challenged with 100 PNU of ragweed allergen or timothy grass (depending on the “off season” allergen) per segment. The allergen preparations at the concentrations used were all below the United States Pharmacopeial acceptable limit of endotoxin for injection (0.25 endotoxin units/mL) as determined by the limulus amoebocyte lysate assay.²³

Cell Collection

The BAL cells were obtained by fiberoptic bronchoscopy and processed as previously described.²⁴ Differential cell counts were obtained from cytospins stained with a modified Wright stain. Mean viability of BAL cells was greater than 95% as determined by trypan blue dye exclusion.

Immunocytochemistry

PPAR- γ protein expression was evaluated in cytospin preparations from freshly isolated BAL cells by immunocytochemistry. Cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and stained with rabbit polyclonal anti-PPAR- γ (1:1,000; Santa Cruz Biotechnology Inc, Santa Cruz, CA), followed by ALEXA-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Slides were also stained with propidium iodine to facilitate nuclear localization, evaluated by confocal microscopy, and quantified by the Image-Pro Plus version 5.0 (MediaCybernetics, Silver Spring, MD). A minimum of 4 to 6 high-power fields were quantified per slide. Mean fluorescence per cell was quanti-

fied for 40 to 100 cells per specimen, and only cells with the morphologic appearance of alveolar macrophages were quantified.

RNA Purification and Analysis

Total RNA was extracted from BAL cells by RNeasy protocol (Qiagen, Valencia, CA). Expression of messenger RNA (mRNA) was determined by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) using the ABI Prism 7700 Detection System (TaqMan; Applied Biosystems, Foster City, CA). Primers and probes were designed using Primer Express software (Applied Biosystems). Reactions were set up according to the instructions of the manufacturer. Briefly, a 125- μ L reaction mixture that consisted of 25 μ L of 5 times Taqman EZ buffer, 15 μ L of 25mM Mn(Oac)₂ solution, 3.75 μ L each of nucleotide triphosphates (adenosine, cytosine, guanine, uridine), 5 μ L of Taq DNA polymerase, 1.25 μ L of AmpErase uracil N-glycosylase, 0.25 μ L each of the 5' primer, 3' primer, and gene-specific probe (100 μ M each), 2.5 μ L/L of RNA sample (50 ng/mL), and 60.5 μ L of diethyl pyrocarbonate–treated water was prepared. Experiments were performed in duplicate with a 50- μ L reaction mixture per well for the 96-well plate. Standard curve was set up using a 5 times series dilution of an RNA sample and glyceraldehyde 3-phosphate dehydrogenase expression as a reference. Thermal profile of the RT-PCR reaction was performed in 3 stages: stage 1 was at 50°C for 2 minutes followed by 60°C for 30 minutes, stage 2 was at 95°C for 5 minutes, and stage 3 was at 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Data are shown as relative expression after normalizing to the internal glyceraldehyde 3-phosphate dehydrogenase expression levels.

Statistical Analysis

Data were analyzed with the *t* test using Prism software (GraphPad Inc, San Diego, CA). Statistical significance was defined as $P \leq .05$.

RESULTS

Inflammatory Cells

At baseline, the BAL samples from healthy controls and asthmatic patients were not different in viability or cellular differential (Table 1). The allergen-challenged segment from the asthmatic group had an increase in the percentage of

eosinophils ($P = .04$). Both healthy controls and asthmatic patients had increased numbers of polymorphonuclear leukocytes. Almost all of the asthmatic patients and healthy control subjects demonstrated a rise in neutrophil levels, which is consistent with other reports demonstrating an increase in neutrophils at 24 hours and a decline at 48 hours after challenge; in contrast, eosinophil levels peak at 48 hours.^{23,25} The current study participants were evaluated at 24 hours to examine gene expression. Total recovered cells from BAL in the asthmatic patients and the healthy controls were not different at baseline. Postchallenge cell numbers were increased in both groups.

Reduction of PPAR- γ mRNA Expression in BAL Cells After Segmental Allergen Challenge

In allergic asthmatic patients ($n = 9$), PPAR- γ gene expression decreased significantly from baseline to postchallenge BAL (median decrease, 45%; $P = .008$) (Figure 1). For nonatopic controls ($n = 6$), the same measurement was not significantly altered. The baseline PPAR- γ expression in healthy controls and asthmatic patients was not significantly different. To determine which of the BAL cells expressed PPAR- γ protein, we evaluated PPAR- γ protein by immunofluorescence in BAL cells of asthmatic patients before and after allergen challenge. As shown in Figure 2A, the PPAR- γ nuclear expression was significantly decreased in alveolar macrophages from a patient after challenge. The PPAR- γ mRNA expression decreased 64% between baseline and after challenge in this patient. Also of interest was the observation that no nuclear staining was detected in either eosinophils or polymorphonuclear leukocytes, although eosinophils displayed cytoplasmic autofluorescence. Quantification of nuclear PPAR- γ staining demonstrated greater staining in prechallenge alveolar macrophages compared with postchallenge alveolar macrophages ($P = .001$) (Fig 2B). Cytospins evaluated with secondary antibody alone showed no evidence of nonspecific binding (data not shown).

DISCUSSION

The novel findings presented herein show for the first time to our knowledge that PPAR- γ , which is highly expressed in alveolar macrophages of healthy individuals, is significantly reduced in asthmatic patients after segmental allergen challenge, a widely used model of acute airway inflammation in

Table 1. Mean $\pm$ SD Leukocytes in Bronchoalveolar Lavage Fluid After Segmental Allergen Challenge

	Asthmatic patients		Controls	
	Baseline control	24-Hour allergen challenge	Baseline control	24-Hour allergen challenge
Alveolar macrophages, %	93 $\pm$ 2	55 $\pm$ 7	93 $\pm$ 2	64 $\pm$ 11
Lymphocytes, %	5 $\pm$ 1	2 $\pm$ 2	4 $\pm$ 2	2 $\pm$ 1
Neutrophils, %	2 $\pm$ 1	30 $\pm$ 7	3 $\pm$ 2	31 $\pm$ 10
Eosinophils, %	1 $\pm$ 0	14 $\pm$ 6	0	4 $\pm$ 1
Total No. of cells, $\times 10^{*4}$	10.6 $\pm$ 2.4	19.2 $\pm$ 7.4	9.8 $\pm$ 1.9	18.0 $\pm$ 5.4

*Per milliliter of bronchoalveolar lavage fluid.

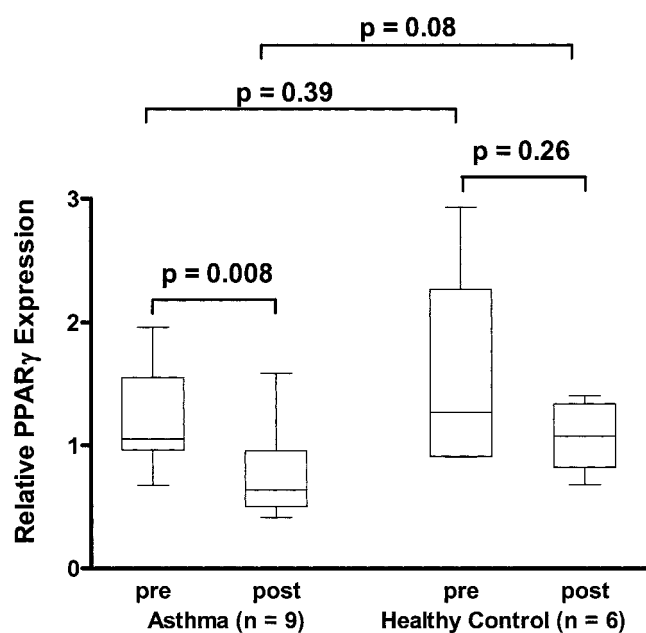


Figure 1. Peroxisome proliferator-activated receptor γ (PPAR- γ) gene expression is decreased in allergic asthmatic bronchoalveolar lavage (BAL) cells after allergen challenge compared with baseline. PPAR- γ messenger RNA was measured in BAL cells from healthy controls ($n = 6$) and allergic asthmatic patients ($n = 9$) at baseline and 24 hours after allergen challenge. The data are shown in a box and whiskers plot. The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value up to the largest.

vivo. Results implicate down-regulation of PPAR- γ in alveolar macrophages as a potential contributor to airway inflammation and dysregulation of lung homeostasis in asthmatic patients.

PPAR- γ expression is ubiquitous within the lung and has been shown to be constitutive in human airway epithelium,^{9,10} alveolar macrophages,^{9,12,13} and smooth muscle cells.¹⁴ PPAR- γ represents a potent transcriptional regulator of genes governing lipid and glucose metabolic pathways in adipocytes, macrophages, and other cell types.^{4,26} The PPAR- γ agonists of the thiazolidinedione class are currently considered to be of greatest importance for treatment of type 2 diabetes mellitus. The discovery that thiazolidinediones have potent anti-inflammatory properties suggests that PPAR- γ activation may also be an effective therapeutic approach to treating inflammatory diseases in the nondiabetic population. Diseases where suggestive data have been obtained include atherosclerosis, psoriasis, atopic dermatitis, and ulcerative colitis.⁵

The present study has several obvious limitations. The segmental allergen challenge is an experimental model only, and the relationship to endogenous spontaneous asthma is unknown. Allergic asthmatic patients in the present study were selected on the basis of their immediate asthmatic airway response (a decrease of $>20\%$ FEV₁ within incremental aerosolized whole lung allergen challenge).

Several murine models of airway hyperreactivity have suggested attenuation of symptoms after treatment with PPAR- γ ligands (ciglitazone, rosiglitazone),^{17,18,27} including reduced eosinophilia. Furthermore, the PPAR- γ -specific antagonist GW9662 prevented the reduction in eosinophilia.¹⁸ Interestingly, therapeutic administration of PPAR- γ ligands to 2 diabetic asthmatic patients appeared to produce a beneficial effect on their asthma.²⁸ Thus, PPAR- γ ligands may represent a novel addition to asthma therapeutics.

Whether other isoforms of PPAR (α , δ) play a role in human allergic asthma has not been explored. However, murine models suggest that although PPAR- α agonists may also be effective,^{19,20} PPAR- δ agonists were not.²⁰ These observations may be related to concentration of the PPAR

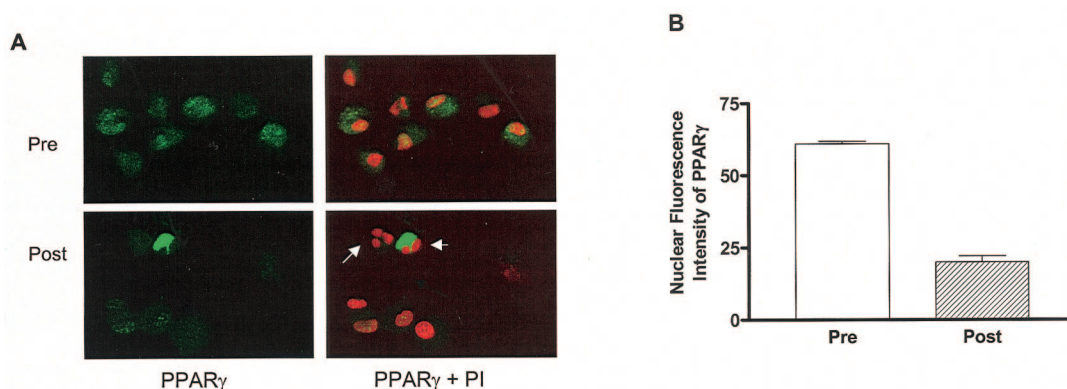


Figure 2. A, Peroxisome proliferator-activated receptor γ (PPAR- γ) nuclear expression is decreased in bronchoalveolar lavage (BAL) cells from an asthmatic patient. Cytospin preparations from BAL cells obtained at baseline and 24 hours after allergen were immunostained with fluorescein isothiocyanate anti-human PPAR- γ and evaluated for immunofluorescence (green) with and without the nuclear stain propidium iodine (PI) (red). Note the polymorphonuclear leukocyte and the eosinophil (marked by arrows) have no nuclear staining. Images were captured at $\times 400$ magnification. B, Immunoquantification demonstrated a 67% reduction in nuclear fluorescence.

isoform. Asada et al¹³ have shown that expression of PPAR- γ is much higher than PPAR- δ expression in human alveolar macrophages.

PPAR- γ agonists have been shown to inhibit the production of numerous inflammatory cytokines, including tumor necrosis factor α , interleukin 1 β , interleukin 6, RANTES, and macrophage inflammatory protein 1 α , all of which have been associated with asthmatic exacerbations. Most of the anti-inflammatory properties of PPAR- γ are thought to arise through their ability to antagonize NF- κ B, AP-1, and STAT-1 signaling pathways.⁴ Thus, many of the cytokines implicated in asthma having NF- κ B elements could potentially be regulated by PPAR- γ agonists. Also of interest with regard to asthma are MMPs.^{1,29} MMPs are implicated in asthma pathogenesis because of their effects on matrix deposition and degradation and their influence on migration of inflammatory cells.²⁹ Our previous studies have demonstrated that both MMP-9 and MMP-12 are elevated in asthmatic patients after challenge compared with baseline.³⁰ Both MMP-9 and MMP-12 are negatively regulated by PPAR- γ activation,^{6,31} suggesting that in asthma reduced PPAR- γ may exacerbate MMP actions.

In summary, the findings from this study demonstrate reduced expression of PPAR- γ in an experimental model of allergic airway inflammation in a group of mild asthmatic patients compared with controls. In the context of provocation-induced asthmatic airway inflammation, PPAR- γ was reduced, suggesting an inverse relationship between PPAR- γ and airway inflammation. Together with in vitro studies that support an anti-inflammatory role for PPAR- γ and the beneficial effects of PPAR- γ agonists in attenuating murine models of airway hyperreactivity, our studies represent important in vivo data to further support the role of PPAR- γ in asthma and that PPAR- γ ligands offer a novel therapeutic approach.

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