Airway epithelial cell PPARγ modulates cigarette smoke-induced chemokine expression and emphysema susceptibility in mice

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Solleti SK, Simon DM, Srisuma S, Arikan MC, Bhattacharya S, Rangasamy T, Bijli KM, Rahman A, Crossno JT, Jr, Shapiro SD, Mariani TJ. Airway epithelial cell PPARγ modulates cigarette smoke-induced chemokine expression and emphysema susceptibility in mice. Am J Physiol Lung Cell Mol Physiol 309: L293–L304, 2015. First published May 29, 2015; doi:10.1152/ajplung.00287.2014.—Chronic obstructive pulmonary disease (COPD) is a highly prevalent, chronic inflammatory lung disease with limited existing therapeutic options. While modulation of peroxisome proliferator-activating receptor (PPAR)-γ activity can modify inflammatory responses in several models of lung injury, the relevance of the PPARγ pathway in COPD pathogenesis has not been previously explored. Mice lacking Pparγ specifically in airway epithelial cells displayed increased susceptibility to chronic cigarette smoke (CS)-induced emphysema, with excessive macrophage accumulation associated with increased expression of chemokines, Cxcl10, and Cxcl15. Conversely, treatment of mice with a pharmacological PPARγ activator attenuated Cxcl10 and Cxcl15 expression and macrophage accumulation in response to CS. In vitro, CS increased lung epithelial cell chemokine expression in a PPARγ activation-dependent fashion. The ability of PPARγ to regulate CS-induced chemokine expression in vitro was not specifically associated with peroxisome proliferator response element (PPRE)-mediated transactivation activity but was correlated with PPARγ-mediated transrepression of NF-κB activity. Pharmacological or genetic activation of PPARγ activity abrogated CS-dependent induction of NF-κB activity. Regulation of NF-κB activity involved direct PPARγ-NF-κB interaction and PPARγ-mediated effects on IKK activation, IkBα degradation, and nuclear translocation of p65. Our data indicate that PPARγ represents a disease-relevant pathophysiological and pharmacological target in COPD. Its activation state likely contributes to NSB-dependent, CS-induced chemokine-mediated regulation of inflammatory cell accumulation.

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is now the third leading cause of death in the United States, and is predicted to become the third most common cause of death in the world by the year 2020. COPD is characterized by progressive development of airflow limitation that is not fully reversible (56), associated with inflammation, small airways thickening, and abnormal enlargement of airspace distal to the terminal bronchioles resulting from destruction of lung parenchyma (emphysema). Cigarette smoke (CS) is the major environmental risk factor for the development of COPD in the United States (18, 31) (86). Smoke-induced emphysema is associated with an increase of inflammatory cells, such as macrophages, neutrophils, dendritic cells, and T lymphocytes, in the lungs (12, 50, 77). Studies have confirmed the presence of persistent inflammation in COPD patients even following smoking cessation (27, 46, 80). With the enormous burden of disease and persistent increase in COPD prevalence, the identification of novel therapeutic targets for disease-modifying therapy has recently gained heightened interest.

Peroxisome proliferator-activated receptors (PPARs) belong to the ligand-dependent nuclear hormone receptor superfamily (10), and are classified into isoforms α, β (or δ), and γ. PPARs function as heterodimers with the 9-cis retinoic acid receptor (RXR). Ligand-activated PPAR-RXR heterodimers bind to peroxisome proliferator response elements (PPREs) on target gene promoters, regulate gene transcription, and control a wide range of cellular functions (10). PPARγ has several naturally occurring agonists, such as arachidonic metabolites, and is also a known target of the insulin-sensitizing, antidiabetic class of pharmaceuticals termed the thiazolidinediones (TZDs) (10).

Pleiotropic effects of PPARs include lipid and lipoprotein metabolism and adipogenesis, glucose homeostasis, cell cycle regulation, and cellular proliferation and differentiation. PPARγ has been found in various cells of the immune system, endothelium, epithelial cells, fibroblasts, glial cells, and smooth muscle cells (10, 22, 17, 55, 72, 74). Multiple studies demonstrate a role for Pparγ in the pathogenesis of inflammation and immune diseases (4, 19, 26, 41, 79, 81, 91). In the lung, Pparγ suppress morbidity in models of asthma, acute lung injury, and fibrosis (72, 84). Anti-inflammatory properties of the PPARγ include the potential to interfere with transcriptional pathways involved in inflammatory responses, such as modulation of NF-κB signaling. Several mechanisms for PPARγ-mediated regulation of NF-κB function have been...
described, including inhibition of IkBα degradation, reduction of RelA (p65) nuclear translocation, and diminished binding of RelA to the DNA (43, 82).

We previously reported the expression of PPARγ is prominent in the airway epithelium of mouse lungs (69). In an effort to define the physiological role of PPARγ within the lung epithelial cells, we generated an airway epithelial-specific conditional Pparg-deficient mouse (69) and showed that PPARγ-targeted mice display nonprogressive nondestructive, congenital airspace enlargement. However, these mice were healthy and did not show any features of inflammation and emphysema. In this present investigation, we show increased susceptibility to CS-induced emphysema in mice lacking PPARγ specifically in the airway epithelium, suggesting deficiency or decreased activity of PPARγ could be a risk factor for COPD. Importantly, we show pharmacological PPARγ activation protects mice from smoke-induced inflammation in vivo, including the attenuation of cellular and molecular intermediates of emphysema pathogenesis in humans. Our data further indicate that Pparg induction in epithelial cells represents a protective mechanism to CS-induced injury response, where it may function to suppress NF-κB-mediated proinflammatory chemokine expression in an activation-dependent fashion.

MATERIALS AND METHODS

Materials. Penicillin/streptomycin, fungizone, t-glutamine, nonessential amino acids, heat-inactivated FBS, Dulbecco’s Modified Eagle Medium (DMEM), RPMI 1640, and phosphate-buffered saline were purchased from Invitrogen (Carlsbad, CA). Rosiglitazone potassium salt (RGZ) and Azaeyal PAF (AzAPAF) were purchased from Cayman Chemicals (Ann Arbor, MI), and 10 μM stock solutions were prepared by dissolving in DMSO and stored at −20°C. Other materials were from the following sources: HEPES (H-4034; Sigma, St. Louis, MO); 10% buffered formalin (Fisher, Pittsburgh, PA); 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA); xylene (Fisher); Harris hematoxylin (Sigma); Gill’s No. 3 hematoxylin (Sigma); cigarette smoke condensate (CSC; Murty Pharmaceutical, Lexington, KY), Nonidet P-40 (MP Biomedicals, Solon, OH); NaCl (JT Baker); EDTA (Promega, Madison, WI); EGTA (JT Baker); DTT, (Invitrogen Life Technologies, Grand Island, NY); Vectastain elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA); Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products, Deerfield, IL); bicinechonic acid (BCA) kit (Pierce, Rockford, IL); Trizol (Invitrogen, Madison, WI); DNA-free (Ambion, Austin, TX); antibodies against p-IκKα/β (Ser70); sc-21661; IkBα (C-21): sc-371, NF-κB p65 (A): sc-109, NF-κB p50(E-10): sc-8414, actin (I-19)-R: sc-1616R, PAPRγ (H-100): sc-7196 (Santa Cruz Biotechnology, Santa Cruz, CA); goat antirabbit or antimouse IgG HRP conjugate (Sigma); protein A/G plus: sc-2003 agarse beads (Santa Cruz Biotechnology); Protran BA 83 nitrocellulose membrane (GE Healthcare, Pittsburgh, PA); protease inhibitor cocktail, phosphatase inhibitor cocktails 2 and 3 (Sigma); rat anti mouse Mac-3 antibodies (BD Pharmingen, San Jose, CA); Lipofectamine 2000 (Invitrogen Life Technologies); and ECL plus Western blotting detection system (GE Healthcare).

Animals. All animal care and treatment procedures were performed in adherence to the National Institutes of Health guidelines and approved by the University of Rochester Committee on Animal Resources. All mice were housed in a pathogen-free vivarium facility of the University of Rochester with a 12:12-h light-dark cycle. The generation and maintenance of airway epithelial-specific Pparg-targeted mice were previously described in detail by Simon et al. (69). Briefly, lung epithelium-specific PPARγ-deficient mice were generated by breeding the CCMCre transgenic line with mice harboring loxp sites flanking exon 2 of the PPARγ gene (PPARγ-floxed mice) (1). Mice were maintained in a mixed C57BL6/129SV background. Conditionally targeted animals have PPARγfloxed/floxed Cre−/− genotype, and littermate control animals have PPARγfloxed/floxed Cre−/+ genotype.

Smoke exposure. All animal procedures were performed with approval and in accordance with institutional guidelines. Twelve-week-old airway epithelium-specific Pparg-targeted and wild-type (WT) littermate control mice (n = 6), maintained in a mixed C57BL6/129SV background, were subjected to the smoke from two nonfiltered cigarettes each day (University of Kentucky research cigarettes), 6 days/wk for 6 mo, essentially as previously described by Hautamaki et al. (36). Age-matched control animals were exposed to filtered room air.

Isolation of whole lung protein. One whole lobe of the mouse lung tissue was homogenized in 0.5 ml of HEPES buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 1 mM DTT containing protease inhibitors). Nonidet P-40 was added to 0.5%, and the homogenates were vortexed and kept on ice for 15–20 min. Supernatants were collected by centrifugation at 10,000 g for 10 min. Protein concentrations were measured by the BCA method. Protein concentrations were measured by the BCA method.

Lung histology and morphometry. Mice were killed by CO2 narcosis, the lungs were lavaged with 3 vol (1 ml/25 g) of ice-cold PBS, and the pulmonary vasculature was perfused with PBS. Three lobes of the right lung were secured individually with suture; the tissue was resected and flash-frozen in liquid nitrogen for RNA, protein, and DNA isolation. The left lung was inflated to a fixed pressure of 25 cmH2O with 10% buffered formalin for 15 min. The inflated lung was removed en bloc and immersed in 35 ml 10% buffered formalin for 48 h at room temperature (RT) or in 4% paraformaldehyde for 24 h at 4°C. After fixation, the tissue was embedded in paraffin for histological and morphological analysis.

Lung sections were deparaffinized and rehydrated by passing through a series of xylene and graded alcohol and were stained with a modified Gill’s hematoxylin (1:1 Harris hematoxylin and Gill’s No. 3 hematoxylin) overnight at RT. The slides were washed and soaked in dilute ammonium hydroxide for 5 min and then dehydrated through ethanol and washed in xylene before coverslipping. Assessment of emphysema was determined essentially as we previously described (69, 70). Briefly, 15 representative images were captured from each slide using a motorized Zeiss microscope with ×10 objective. For each lung image, alveolar size was estimated from the mean linear intercept (Lm) of the airspace, which is a measure of airspace enlargement/emphysema using computer-assisted morphometry using the Metamorph software program.

Bronchoalveolar lavage phenotyping and immunohistochemistry. Immediately following exposure to CS, mice (n = 4–6/group) were killed by CO2 narcosis and lavaged with 3 vol (1ml/25 g) of ice-cold PBS by inserting a catheter in the trachea. The bronchoalveolar lavage (BAL) fluid collected from the lungs of the mice was centrifuged (3,000 rpm for 3 min at 4°C), and the supernatant was retained for further analysis. The red blood cells in cell pellets were lysed, and BAL cells were resuspended in 1.0 ml PBS. Cells were centrifuged (Shandon Southern Products) on glass slides and stained with Wright-Giemsa stain to evaluate cellular content. Alternatively, lung macrophages were analyzed using Mac-3 (1:1,000 dilution) immunostaining on midsagittal sections using the Vectastain elite avidin-biotin complex kit as per the manufacturer’s instructions. The number of Mac-3-positive cells in the lung sections (n = 4/group and 10 fields/lung section) were counted manually.

RNA isolation and real-time quantitative RT-PCR. Lung tissue samples were homogenized in TRIzol, and total RNA was extracted according to the manufacturer’s protocol. Tissue RNA was repurified and rendered DNA-free, and RNA was isolated from cultured cells, using the Absolutely RNA micro prep kit (Stratagene, La Jolla, CA). RNAs were reverse-transcribed using the Gene Amp RNA-PCR kit (Applied Biosystems). Quantitative real-time PCR (qPCR) analysis was performed using the Mx3000p Detection System (Stratagene).
using TaqMan or SYBR Green chemistry (Applied Biosystems). Gene expression levels were calculated relative to *P. sia* (cyclophilin A) using the ddCT method as we have previously described (73). Primer sequences are listed in online supplemental Table E1.

Proinflammatory mediator analysis. The levels of CCL5 and CXCL10/IFN-γ inducible protein 10 (IP-10) in H292 cells and the levels of CCL5, CXCL8, and CXCL10 in the basolateral supernatant of primary normal human bronchial epithelial (NHBE) cell cultures were measured by Luminex xMAP technology (Beadlyte; Millipore, Temecula, CA). The levels of Cxcl10 protein in mouse lung homogenates were measured by ELISA using duo-Ab kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

In vivo drug treatment. Twelve-week-old female WT C57BL/6J mice (*n* = 4–5 animals/treatment group) were exposed to up to 12 wk of CS while continuously fed a normal, control diet or the same diet impregnated with PPARγ-activating TZD ligands RGZ (15 mg·kg⁻¹·day⁻¹) or pioglitazone (PIO; 33 mg·kg⁻¹·day⁻¹). Food (Diet) and water were provided every other day, ad libitum, while mice were maintained under the 12-h light cycle (21).

Cell culture. The cell lines used in our studies included NCI-H292 cells (kindly provided by Dr. Irfan Rahman, University of Rochester), an immortalized human pulmonary mucoepidermoid carcinoma cell line, and a mouse transformed Club cell (mTC) line (kindly provided by Dr. Robert Senior, Washington University). H292 were maintained in RPMI 1640 medium, and mTC were maintained in DMEM medium. Media were supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (1 µg/ml), and cells were placed in a humidified incubator at 5% CO2 at 37°C. Experimental treatments with CSC or RGZ, alone or in combination, were provided fresh serum-free medium containing the appropriate treatment conditions. For ectopic overexpression experiments, transfected cells were refed with fresh medium for 24 h before treatment. Luciferase activity was assessed 48 h after the treatment using the Promega luciferase assay system and the β-galactosidase enzyme assay system (Promega).

Cell lysis, immunoprecipitation, and immunoblotting. Cells were washed twice in ice-cold PBS, lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mm Tris·HCl, pH 7.4, 150 mm NaCl, 5 mm NaF, 0.25 mm EDTA, pH 8.0, 1% deoxycholic acid, and 1% Triton X-100) supplemented with protease inhibitor cocktail and phosphate inhibitor cocktails 2 and 3, and stored at −80°C. The protein concentration of the extract was measured using the BCA method.

For immunoprecipitation, 300–500 µg protein were subjected to preclearing with 10 µl of protein A/G-agarose beads for 1 h at 4°C. The precleared cell lysates were subjected to immunoprecipitation by incubating with 0.6–1.0 µg of PPARγ antibodies and 10–20 µl of the protein A/G-agarose beads at 4°C overnight with gentle agitation. The immunoprecipitated samples were washed four times with 1 ml of ice-cold RIPA followed by two washes with ice-cold PBS. The proteins in the immunoprecipitates were extracted by boiling with SDS sample buffer for 10 min. Cell lysates or extracted proteins (10–40 µg) were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The membranes were blocked by incubation with 5% (wt/vol) nonfat dry milk in 10 mm Tris, pH 8.0, 150 mm NaCl, and 0.05% Tween 20 for 1 h at RT. The membranes were then incubated with appropriate primary and secondary antibodies and developed using an ECL kit as per the manufacturer’s instructions. Western blotting of nuclear protein was used to determine nuclear translocation of p65 and p50, and cytoplasmic protein was used to determine IκBα degradation. For detecting phosphorylation of IKK, total cell lysate was used.

Statistical analysis. All experiments were repeated at least twice with multiple replicates, and data are expressed as means ± SE. Comparisons between experimental groups were made by either the parametric Student’s *t*-test or nonparametric Mann-Whitney *U*-test. Differences in mean values were considered significant at *P* < 0.05.

RESULTS

Epithelial cell-specific deletion of *pparg* aggravates susceptibility to smoke-induced emphysema in mice. Airway epithelial cells are known to play a physiological role in regulating lung inflammation (71, 78). We previously described a line of conditionally targeted mice lacking *Pparg* in a majority of their lung airway epithelial cells (69). These mice display mild, congenital airspace enlargement that is not associated with tetrypsinization, and resuspended in 400 µl of 10 mm HEPES pH 7.9, 10 mm KCl, 0.1 mm EGTA, 0.1 mm EDTA, and 1 mm DTT supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. After 15 min, Nonidet P-40 was added to a final concentration of 0.6%. Samples were centrifuged to collect the supernatants containing cytosolic proteins. The pelleted nuclei were resuspended in 100 µl of 20 mm HEPES, pH 7.9, 0.4 mm NaCl, 1 mm EDTA, 1 mm EGTA, and 1 mm DTT supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. After 30 min incubation at 4°C, lysates were centrifuged, and supernatants containing the nuclear proteins were collected, snap-frozen, and stored at −80°C. The protein concentration of the extract was measured using the BCA method.

Reporter assays. H292 cells were transfected with luciferase reporter and expression plasmids using Lipofectamine 2000 according to the manufacturer’s instructions, using either the PPRE or NF-κB luciferase reporter constructs (0.8–1.44 µg) and PPARγ overexpression construct (0.8 µg) along with 0.16 µg pCMV-LacZ construct or pRL-SV40 construct. After 12 h of transfection, cells were provided fresh serum-free medium containing the appropriate treatment conditions. For ectopic overexpression experiments, transfected cells were refed with fresh medium for 24 h before treatment. Luciferase activity was assessed 48 h after the treatment using the Promega luciferase assay system and the β-galactosidase enzyme assay system (Promega).

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tissue inflammation or parenchymal destruction, and is not progressive with age (69). To test the hypothesis that loss of PPARγ function in the airway epithelium would result in increased susceptibility to COPD, airway epithelial cell Pparγ-targeted mice were challenged with chronic CS exposure. Histopathological analysis of lungs of targeted mice exposed to 6 mo of CS revealed features of emphysema pathology (Fig. 1A). Quantitative analysis revealed a significant increase ($P < 0.01$) in the two-dimensional sizes of individual airspaces (airspace area) in CS-exposed targeted mice compared with nonexposed littermate controls (50% increase, $P < 0.01$). These results indicate that loss of PPARγ function specifically in epithelial cells results in an exaggerated inflammatory response associated with increased emphysema pathology upon chronic exposure to CS.

CS-induced emphysema is associated with exacerbated chemokine expression in conditionally targeted pparγ knockout mice. Airway epithelial cells can play a physiological role in controlling lung inflammation and emphysematous pathology (7), particularly through the regulation of inflammatory cell-recruiting chemokine production. We examined the expression of chemokines in the lungs of CS-exposed mice. Whole lung tissue qPCR analysis revealed that CS exposure increased the expression Ccl5, Cxcl10, and Cxcl15 (Fig. 2A) compared with age-matched, room air-exposed mice. Loss of Pparγ expression in targeted mice resulted in the exaggerated induction expression of Ccl5 (1.3 vs. 1.9 vs. 1.1-fold), Cxcl10 (2.7 vs. 1.2 vs. 8.0 vs. 5.3-fold, $P < 0.05$), and Cxcl15 (8.6 vs. 3.8 vs. 13 vs. 4.7-fold) compared with smoke-exposed littermate controls.

Previous reports have indicated increased chemokine expression, including Cxcl10, is associated with COPD in humans (33, 52, 63). We demonstrated increased bioavailability of Cxcl10 protein in the lungs of CS-exposed WT mice (0.8 vs. 3.8 ng/mg protein, $P < 0.05$), which was further augmented in targeted mice (3.8 vs. 5.5 ng/mg, $P = 0.09$) (Fig. 2B). These results indicate increases in chemokine expression are associated with enhanced macrophage accumulation and emphysema susceptibility in mice lacking airway epithelial cell PPARγ.
Pharmacological activation of PPARγ reduces smoke-induced inflammation in mice. PPARγ-activating drugs have shown anti-inflammatory effects in various animal models of lung injury (11, 28, 87). To test the potential therapeutic benefit of PPARγ activation in the context of COPD, adult WT C57BL/6 mice were fed a control diet, or the same diet impregnated with 15 mg/kg RGZ or 33 mg/kg PIO, while exposed to CS for up to 12 wk. CS exposure resulted in a significant increase (1.7-fold, \( P < 0.05 \)) (Fig. 3A) in the accumulation of total inflammatory cells recoverable by BAL, and a significant increase in the accumulation of macrophages (1.6-fold, \( P < 0.05 \)) (Fig. 3B), which represented 90% of total cells. PIO treatment resulted in a significant reduction in the total number of inflammatory cells (1.5-fold, \( P < 0.05 \)) (Fig. 3A) and macrophages (1.5-fold, \( P < 0.05 \)) (Fig. 3B) recoverable by BAL in smoking mice. Similar results were obtained using RGZ as a therapeutic activator of PPARγ. CS also resulted in a significant increased number of lymphocytes (6-fold, \( P < 0.05 \)) (Fig. 3D) and a nonsignificant increase in neutrophil number (1.8-fold) (Fig. 3C), neither of which showed a significant decrease when smoke-exposed mice were treated with RGZ (Fig. 3D).

Next, we tested whether the reduction in inflammatory cell accumulation in TZD-fed, smoke-exposed mice was associated with changes in chemokine expression. CS exposure for 12 wk induced the expression of lung Cxcl10 and Cxcl15 at the RNA level (Fig. 3E). RGZ treatment abrogated CS-induced expression of Cxcl10 (2- vs. 0.3-fold, \( P < 0.05 \)) and Cxcl15 (9.5- vs. 0.4-fold, \( P < 0.05 \)) compared with mice on a control diet. The ability of RGZ treatment to significantly inhibit Cxcl10 expression in CS-exposed mice was also confirmed at the protein level (5.2 vs. 3.5 ng/mg, \( P < 0.05 \)) (Fig. 3F). These data indicate that therapeutic activation of PPARγ activity, using either of two specific pharmacological PPARγ ligands, can limit lung chemokine-associated inflammation in response to CS exposure.

PPARγ regulates CS-induced chemokine expression in cultured lung epithelial cells. Previous reports suggest that CS exposure of epithelial cells in vitro can directly or indirectly recapitulate in vivo injury responses (48, 78). To understand
the cellular mechanisms involved in in vivo observations, we studied the effects of CSC in cultured lung epithelial cells. Treatment of mtCC with CSC (75 µg/ml) modestly induced the mRNA expression of Ccl5, Cxcl10, and Cxcl15 (Fig. 4A). Importantly, PPARγ activation using RGZ treatment (12 µM) was capable of significantly reducing the expression of Ccl5 (1.45- vs. 0.4-fold, \( P < 0.05 \)), Cxcl10 (2.4- vs. 0.8-fold, \( P = 0.05 \)), and Cxcl15 (1.46- vs. 0.9-fold, \( P < 0.05 \)) in the presence of CSC (Fig. 4A). Consistent with these data obtained from murine cells, CSC also significantly induced CCL5 (7.3-fold, \( P < 0.05 \)) and CXCL10 (12.5-fold, \( P < 0.05 \)) expression in H292 human lung airway-like epithelial cells at the protein level (Fig. 4B). Importantly, CSC-induced H292 protein levels of CCL5 (7.3- vs. 0.9-fold, \( P < 0.05 \)) and CXCL10 (12.5- vs. 1.1-fold, \( P < 0.05 \)) were also abrogated by RGZ treatment.

We investigated the specificity of these responses using non-TZD, PPARγ-agonists, to modulate CCL5 and CXCL10 expression at the protein level. Similar to RGZ, AzPaf (1 µM) also abrogated CSC-induced CCL5 (7.3- vs. 0.8-fold, \( P < 0.05 \)) and CXCL10 (12.5- vs. 0.7-fold, \( P < 0.05 \)) (Fig. 4B) protein levels, suggesting the PPARγ activation-dependent nature of these ligands for regulating chemokine suppression.

We next tested the effects of CSC and TZD treatment upon chemokine expression in NHBE cells differentiated at ALI. Treatment of NHBE cells with CSC (40 µg/ml) (Fig. 4, C and D) resulted in significant increases in mRNA levels of CCL5 (1.8-fold, \( P < 0.05 \)) and CXCL8 (1.7-fold, \( P < 0.05 \)), but not CXCL10. Importantly, CSC treatment resulted in large and significant increases in the basolateral secretion of CCL5 (34-fold, \( P < 0.05 \)), CXCL10 (21-fold, \( P < 0.05 \)), and CXCL8 (26-fold, \( P < 0.05 \)). Conversely, RGZ treatment (12 µM) significantly abrogated the mRNA levels of CCL5 (1.8- vs. 0.88-fold, \( P < 0.05 \)) and CXCL8 (1.7- vs. 0.67-fold, \( P < 0.05 \)) (Fig. 4C), and protein levels of CCL5 (34- vs. 2.7-fold, \( P < 0.05 \)), CXCL10 (21- vs. 1.3-fold, \( P < 0.05 \)), and CXCL8 (26- vs. 1.2-fold, \( P < 0.05 \)) (Fig. 4D). These results confirm that PPARγ activation regulates and abrogates smoke-induced chemokine expression in lung epithelial cells.

**Pharmacological activation of PPARγ suppresses smoke-induced NF-κB activity in lung epithelial cells.** NF-κB is a master regulator of proinflammatory cytokines and chemokine expression (37, 38). PPARγ has been reported to have anti-

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Fig. 4. CS-induced lung epithelial cell chemokine expression is suppressed upon activation of PPARγ. A and B: confluent cultures of lung epithelial cell lines were treated with cigarette smoke condensate (CSC, 75 µg) alone, or in combination with PPARγ-activating ligand, for 48 h. A: gene expression in mouse transformed Club-like (Clara) cells (mtCC) treated with CSC and RGZ (12 µM) was analyzed by qPCR for the genes indicated. B: H292 human lung airway-like epithelial cells treated with CSC, RGZ (12 µM), or Azeloyl PAF (AzPaf, 1 µM) and analyzed for CCL5 and CXCL10 protein levels by xMAP. C and D: primary normal human bronchial epithelial (NHBE) cells were differentiated at the air-liquid interface (ALI) and treated with CSC (40 µg) and RGZ (12 µM) for 48 h. C: gene expression was analyzed by qPCR for the genes indicated. D: protein secretion into the basolateral secretion of CCL5 (7.3- vs. 0.9-fold, \( P < 0.05 \)) protein levels, suggest-
inflammatory effects, and PPARγ ligands have been used in clinical trials for patients with inflammatory diseases (30). Multiple studies have demonstrated that many anti-inflammatory effects of PPARγ are mediated through transrepression of NF-κB in a NCoR/sumoylation-dependent pathway (5, 29, 39, 54).

We tested the effects of PPARγ on NF-κB transcriptional activation by CS in lung epithelial cells. H292 cells were treated with CSC (60 μg/ml) alone, or in combination with RGZ (12 μM), and transcriptional activation was assessed using PPRE-driven (Fig. 5A) or NF-κB element-driven (Fig. 5B) promoter reporter constructs. (PPRE)3-TK-Luc (64) and NF-(κB)5-E1B-Luc (51). As expected, RGZ increased transcriptional activity of the Pparg reporter (1.6-fold vs. untreated; \(P < 0.01\)). CSC alone demonstrated a modest, but significant increase in PPRE activity (1.7-fold vs. untreated; \(P < 0.007\)). Cotreatment with CSC and RGZ had a slight additive effect upon PPRE activity (2.1-fold vs. untreated; \(P < 0.04\)). Conversely, while CSC alone strongly induced NF-κB activity (2.1-fold vs. untreated, \(P < 0.03\)), RGZ significantly reduced both baseline (0.3-fold vs. untreated, \(P < 0.001\)) and CSC-induced (0.6-fold vs. CSC alone, \(P < 0.04\)) NF-κB activity. These data suggest that chemokine expression in lung epithelial cells is correlated with NF-κB activity, but not PPRE activity.

Inhibitory effects on smoke-induced NF-κB activity are robust and dependent upon PPARγ protein. The TZD class of ligands has been shown to function via both PPARγ-dependent and -independent mechanisms (16, 23). We demonstrated the PPARγ-activating activity of a non-TZD-activating ligand (AzPAF) in H292 cells (online supplemental Fig. E2). To confirm that the in vitro effects of RGZ upon NF-κB signaling are PPARγ-dependent, we tested the activity of AzPAF on NF-κB activity in H292 cells (Fig. 6A). Similar to RGZ, treatment with AzPAF (1 μM) inhibited baseline (0.4-fold vs. untreated; \(P < 0.01\)) and CSC-induced (0.4-fold vs. CSC alone, \(P < 0.02\)) NF-κB transcriptional activity.

Next, we tested the ability of ectopic expression of either WT (Fig. 6B) or dominant-negative AF2 domain mutant (Fig. 6C) PPARγ protein upon NF-κB activity in H292 cells. As expected, ectopic expression of WT PPARγ increased baseline PPRE activity, whereas dominant-negative mutant PPARγ reduced baseline PPRE activity (online supplemental Fig. E3). Cotreatment of cells with RGZ had no effect, suggesting the

**Fig. 5.** PPARγ activation suppresses CSC-induced NF-κB transcriptional activity in human lung airway epithelial cells. PPARγ and NF-κB transcriptional activity was measured in human lung epithelial cells (H292), treated with RGZ and/or CSC for 48 h, using reporter assays. A: cells were transiently transfected for 12 h with (PPRE)3-TK-Luc and pCMV-β-gal, followed by treatment with RGZ (12 μM) and/or CSC (60 μg/ml), as indicated. Results are presented as mean relative luciferase units (RLU) normalized to β-gal activity. Treatment with RGZ or CSC increased PPARγ transcriptional activity. B: cells were transiently transfected for 12 h with pNF-(κB)5-E1B-Luc and pCMV-β-gal, followed by treatment with RGZ (12 μM) and/or CSC (60 μg), as indicated. Treatment with CSC increased NF-κB transcriptional activity, whereas RGZ decreased both baseline and CSC-induced, NF-κB activity. Results are presented as mean RLU ± SE normalized to β-gal activity. \(*P < 0.05\) by \(t\)-test.

**Fig. 6.** Regulation of NF-κB activity by nonpharmacological PPARγ ligands or by ectopic PPARγ expression. A: human lung airway epithelial cells (H292) were transiently transfected with pNF-(κB)5-E1B-Luc and then treated with the AzPAF alone, or in combination with CSC (60 μg/ml). Cells were harvested and assayed for normalized luciferase activity (relative to β-Gal) after 48 h. Similar to RGZ, treatment with AzPAF decreased CSC-induced NF-κB transcriptional activation. B: H292 cells were transiently transfected with pNF-(κB)5-E1B-Luc and pSV40-RL along with control vector (V, empty pcDNA3.0), or vector containing a cDNA for wild-type (WT) human PPARγ (V + hPPARγWT). Cells were stimulated with CSC alone (60 μg/ml), or in combination with RGZ (6 μM), and assayed for normalized luciferase activity (relative to β-Gal) after 48 h. Ectopic expression of WT PPARγ suppressed CSC-induced NF-κB activity and potentiated the suppressive effects of RGZ. Results are presented as mean RLU ± SE normalized to β-gal activity. \(*P < 0.05\) by \(t\)-test.
availability of endogenous ligands in unstimulated cells. Expression of WT PPARγ did not induce any significant increase in NF-κB activity at baseline (data not shown). Importantly, ectopic expression of WT PPARγ alone suppressed CSC-induced NF-κB activity (0.6-fold vs. CSC treatment with control vector, P < 0.01), similar to treatment with the PPARγ-activating ligand RGZ. The ability of RGZ treatment, even at low concentrations (6 μM), to suppress NF-κB activity was also enhanced by ectopic expression of WT PPARγ (0.7-fold vs. CSC and RGZ treatment with control vector, P < 0.01). Taken together, these results clearly indicate that the ability of PPARγ ligands to regulate NF-κB activity in lung epithelial cells is PPARγ-dependent.

PPARγ physically interacts with and regulates the NF-κB signaling pathway. The ability of PPARγ to inhibit inflammatory gene expression is, at least in part, due to its ability to directly interact with and inhibit NF-κB in a ligand-dependent and sumoylation-dependent fashion (5). We explored whether CS may affect PPARγ and NF-κB interaction in lung epithelial cells, and if this interaction is associated with NF-κB function. H292 cells were treated with CSC alone (60 μg/ml), or in combination with RGZ (12 μM), followed by coimmunoprecipitation of PPARγ-NF-κB complexes using antibodies against PPARγ. The amount of NF-κB complexed to PPARγ was determined by Western blotting with antibodies specific for p65 (Fig. 7A). While interaction between PPARγ and NF-κB is undetectable in H292 cells under control conditions, CSC treatment resulted in increased coprecipitation. Interestingly, these conditions supported high NF-κB activity (Fig. 5B) and high chemokine expression (Fig. 4). Importantly, RGZ cotreatment with CSC induced the greatest interaction between PPARγ and NF-κB, under conditions where NF-κB activity (Fig. 5B) and chemokine expression (Fig. 4) are suppressed. These results are consistent with prior studies suggesting that PPARγ-activating ligands facilitate interaction with, and are necessary for, inhibition of NF-κB activity.

The predominant form of NF-κB consists of a heterodimer of p50 and p65 subunits that are sequestered in the cytoplasm of unstimulated cells by the inhibitory proteins IκBα and IκBβ (37). Signal-dependent activation of IKK results in phosphorylation and rapid degradation of IκBα and IκBβ, allowing p50/65 heterodimers to translocate to the nucleus. In the nucleus, p50/65 heterodimers bind to and activate numerous proinflammatory genes. To further examine the biochemical mechanisms that underlie PPARγ agonist-induced inhibition of chemokine signaling via NF-κB, we examined whether PPARγ activation affects p65/p50 nuclear translocation. p65 protein was largely localized in the cytoplasm of untreated H292 human lung epithelial cells. A marked increase in nuclear translocation and accumulation of p65 (Fig. 7B), but not p50, was observed upon CSC treatment (60 μg/ml). Interestingly, RGZ (12 μM) reduced both CSC-associated p65 and p50 nuclear translocation (Fig. 7, B and C).

Phosphorylation of IκBα results in its proteolytic degradation, thereby releasing NF-κB dimers for translocation to the nucleus. CSC treatment of H292 cells also induced degradation of IκBα, which was abrogated by cotreatment with RGZ (Fig. 7D). Because stimulus-dependent phosphorylation and activation of IκKα/β (58), the upstream kinase of the NF-κB pathway, led to the degradation of IκBα, we investigated the effects of CS and RGZ on pIκK levels. As shown in Fig. 7E, CSC induced the phosphorylation of IκKα/β, whereas activation of PPARγ by RGZ inhibited the CSC-induced phosphorylation of IκKα/β. These data demonstrate a complex interaction between PPARγ and NF-κB pathways whereby PPARγ activity may act to suppress smoke-induced NF-κB activity in lung epithelial cells at many levels.

**Fig. 7.** PPARγ binds NF-κB and regulates its activity at multiple levels. H292 cells were treated with CSC (60 μg/ml) and RGZ (12 μM), alone or in combination. Cells were harvested for nuclear, cytoplasmic extracts or whole cell extracts after 48 h unless otherwise noted. A: PPARγ and NF-κB complex was coimmunoprecipitated with antibodies against PPARγ, and blotted using p65 antibody. CSC and RGZ treatment promoted the association of PPARγ with NF-κB. B: Cytoplasmic or nuclear extracts were assayed for NF-κB subunits of p65. CSC induced the nuclear translocation of p65. RGZ treatment inhibited the CSC-induced nuclear translocation of p65. C: Cytoplasmic or nuclear extracts were assayed for p50. CSC did not induce the nuclear translocation of p50. RGZ treatment inhibited nuclear translocation of p50. D: Cytoplasmic extracts were assayed for IκBα after 12 h. Whereas CSC-induced degradation of IκBα, RGZ-treatment inhibited the degradation to some extent. E: activation of PPARγ inhibits CSC-induced phosphorylation and activation of IκK. Total protein extracts were separated on 8% gel and immunoblotted using pIκKα/β (Ser173)-specific antibody. β-Actin was used as a protein-loading control. Black line in the gel (A) represents the lanes were run on the same gel but were noncontiguous.
Results from the present study demonstrate the important contribution of airway epithelial cell PPARγ in modulating chronic CS-induced lung injury. Our data demonstrate that ligand-mediated activation of PPARγ transrepression function regulates lung inflammation following chronic exposure to CS. While airway epithelial cell deficiency of PPARγ significantly exacerbated CS-induced pulmonary inflammation and emphysema, pharmacological activation of PPARγ significantly reduced CS-induced lung inflammation.

The anti-inflammatory role of PPARγ was first demonstrated in monocytes/macrophages as an ability to regulate cytokine production, resulting in altered native and acquired immune responses (61). Subsequently, studies of experimental models of inflammatory bowel diseases, periodontitis, renal disorders, and various cancers have supported PPARγ as a potential therapeutic target for both tissue inflammation and carcinogenesis (24, 42, 47, 60, 76). In the lung, augmented PPARγ expression has been detected in airway epithelium, bronchial submucosa, and smooth muscle of asthmatics (9), and in multiple models of allergic and nonallergic lung inflammation. Because complete Pparg deficiency in mice results in embryonic death at midgestation, before lung development (6, 44), animal studies of PPARγ function have primarily used agonists to determine its role. Pharmacological ligand-mediated activation of PPARγ shows a protective effect in many of these lung injury models (8, 45, 53, 66, 85), although the specificity of PPARγ regulation for these responses remains to be determined. PPARγ-specific ligands have been shown to inhibit the production of numerous inflammatory molecules in vivo. Of particular importance in the context of lung inflammation are TNF-α, IL-8, COX2, iNOS, and MCP1 produced by epithelial cells (22, 57, 83, 87). The potential breadth of anti-inflammatory function makes PPARγ an attractive target for lung diseases such as asthma and COPD.

Given the anti-inflammatory effects of PPARγ-activating ligands in other forms of lung inflammation, we sought to test the mechanistic role of epithelial cell PPARγ in COPD. We challenged a previously described airway epithelial cell PPARγ-targeted mouse line (69) with chronic CS exposure. Genetic background of mice strains plays an important role in their susceptibility to CS-induced emphysema (13, 34). These mice were maintained in a mixed C57BL/6/SV129 background, and WT expectedly demonstrated an intermediate response to chronic CS exposure. Regardless, targeting of airway epithelial cell PPARγ led to enhanced airspace enlargement following chronic smoke exposure, implicating the role of PARG in emphysema susceptibility. Although some conditional targeting strategies in mice led to emphysema-like phenotypes, this is associated with expression of reverse tetracycline transactivator protein, which is not present in our transgenic system (59). Furthermore, we have demonstrated that the mice used in this study do not display spontaneous, progressive airspace enlargement or emphysema (69). Consistent with our results, expression of a dominant-negative mutant form of PPARγ in the lung epithelium of mice results in chronic inflammation and airspace enlargement (88). The relevance of this dominant-negative model is not entirely clear, since deficiency in conditionally targeted mice does not result in chronic inflammation in the absence of a “second hit” such as continuous exposure to CS. Nonetheless, an abundance of data from both of these genetic models of PPARγ insufficiency suggests that reduced epithelial PPARγ increases susceptibility to inflammatory responses, including those induced by exposure to CS.

Results from the present study suggest that epithelial cell PPARγ activity contributes to the modulation of inflammatory cell recruitment and emphysema susceptibility in mice. While epithelial cell Pparg deficiency increases emphysema susceptibility, CS-mediated increased PPARγ activity in epithelial cells is likely to be a protective defense mechanism by which PPARγ can act as a resolution or repair pathway against the inflammatory response by CS exposure. In support of this conclusion, the ability of PPARγ to function as an active transcriptional suppressor of NF-κB is correlated with the presence of an activating ligand.

Importantly, targeting airway epithelial cell Pparg led to an increase in critical, disease-relevant cellular (lung macrophage accumulation) and molecular (lung Ccl5, Cxcl10, and Cxcl15 expression) intermediate phenotypes of COPD when challenged with chronic CS exposure. Macrophages are the primary inflammatory cell in the lower airspace under normal conditions, and particularly in response to long-term cigarette smoking (36). Increased infiltration of macrophages, in Pparg-targeted mice, may have contributed to injury to the lung parenchyma through the activity of their elastolytic enzymes, particularly MMP12 (36). CCL5 has been reported to be involved in recruitment of neutrophils in COPD (25). CXCL10 has been reported to be expressed in bronchial epithelium of smokers with COPD, and play a role in attracting T lymphocytes to the site of tissue inflammation (63, 65). Cxcl15 (Lungkine), a novel molecule specifically expressed in airway epithelial cells and induced by inflammatory conditions, recruits neutrophils during pulmonary inflammation (14, 62). Grumelli et al. (33) recently demonstrated that, in human subjects with emphysema, lung macrophages release MMP12 in response to IP-10/CXCL10 and monokine induced by interferon. In total, our studies suggest that PPARγ can modify smoke-induced inflammation in vivo by regulating epithelial cell production of proinflammatory mediators.

Our pharmacological studies demonstrating an effect of exogenous PPARγ activation upon CS-induced inflammation suggest this strategy may have therapeutic benefit. Critically, the loss of PPARγ within the epithelial cells alone increased susceptibility to CS-induced inflammation at the molecular and cellular levels. The effect of PPARγ in nonepithelial cell types within the lung may also be important in regulating inflammatory responses. Shan et al. reported that agonist induction of PPARγ reverses CS-induced emphysema in a myeloid dendritic cell-mediated fashion (67) and PPARγ agonist regulation of CS-induced inflammation in alveolar macrophages reported by Yin et al. (90).

Excessive chemokine expression following smoke exposure in PPARγ-targeted mice could be due to activation of the lung epithelium either directly or indirectly via inflammatory mediators such as TNF-α or IFNγ, which are released in the ongoing process of COPD. Many investigators have modeled the effects of CS on the lungs using extracts or condensates in vitro. For example, TZDs have been reported to decrease CS-induced mucin secretion from human airway epithelial cells in vitro (48). We found that the effects of smoke exposure...
in isolated lung epithelial cells included induction of chemokine expression and activation of NF-κB in vitro in both mouse (mtCC) and human (H292) lung epithelial cells. Importantly, we demonstrated that these effects were specific for PPARγ using multiple pharmacological and genetic activators and inhibitors in vitro. Combined, our in vivo and in vitro results confirm that these epithelial cell responses are dependent upon expression and function of PPARγ, and cannot be explained by nonspecific effects of PPARγ-activating ligands.

PPARγ can interact with various transcriptional factors to regulate inflammatory responses (32). For instance, PPARγ ligands have been reported to inhibit multiple events of the NF-κB pathway (75). Smoke-induced p65 nuclear localization in lung epithelial cells has been reported (3, 68). Our data indicate that PPARγ can regulate NF-κB-dependent signaling at multiple levels. An abundance of evidence suggests that PPARγ exerts many of its physiological anti-inflammatory effects by inhibiting NF-κB through direct binding in a ligand- and SUMOylation-dependent fashion (54). We show that CS activates NF-κB in a PPARγ-dependent manner in vitro. Importantly, the ability of PPARγ activation to inhibit NF-κB was not correlated with PPRE-dependent transcription, but is associated with enhanced physical association with NF-κB.

The airway epithelium has been recognized to participate in various defense functions, including mechanical clearance of offending particles and production of antimicrobial agents. However, critical functions for coordinating lung injury have not been fully appreciated. Lung epithelial cells can secrete proinflammatory mediators in response to various external insults. The airway epithelium is one of the first targets of CS, and increased proinflammatory mediator expression by pulmonary epithelial cells after airway challenge with CSC have been reported (78). Cheng et al. (15) reported that airway epithelium controls the lung inflammatory response and injury through the NF-κB pathway. Results from the present study demonstrate an important anti-inflammatory role of airway epithelial cell PPARγ in regulating smoke-induced lung injury.

Activation of PPARγ by its agonists is known to inhibit the expression of proinflammatory mediators (2, 40, 48, 49). We demonstrated here that pharmacological activation of PPARγ, using TZDs, protects mice from chronic CS exposure in an airway epithelial cell-dependent fashion. Mice fed with RGZ during 12 wk of CS exposure displayed attenuated injury responses as demonstrated by a reduction in both macrophage accumulation and chemokine expression. These data strongly suggest that targeting PPARγ may prove an effective method for alleviating or reversing progressive lung inflammation associated with emphysema. We are currently evaluating the ability of TZDs to block the development of emphysema in mice and suppress lung epithelial cell chemokine expression as part of a phase II clinical trial in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


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