ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 2, 2008

DOI: 10.1089/ars.2007.1889

Forum Original Research Communication

Rosiglitazone and 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2, PPARγ Agonists, Differentially Regulate Cigarette Smoke-Mediated Pro-Inflammatory Cytokine Release in Monocytes/Macrophages

SAMUEL CAITO, SE–RAN YANG, ARUNA KODE, INDIKA EDIRISINGHE, SARAVANAN RAJENDRASOZHAN, RICHARD P. PHIPPS, and IRFAN RAHMAN

ABSTRACT

Peroxisome Proliferator-Activated Receptor gamma (PPARγ) ligands have the potential for use as anti-inflammatory agents in chronic airway diseases. We hypothesized that cigarette smoke (CS)-mediated pro-inflammatory cytokine release would be downregulated in the monocyte–macrophage cell line (MonoMac6) by synthetic and natural PPARγ ligands. Surprisingly, treatment of MonoMac6 cells with the natural PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 led to increased cytokine (IL-8) release in response to either TNF-α or CS extract (CSE). However, exposure to rosiglitazone, a synthetic agonist, led to decreased TNF-α, but not CSE, mediated cytokine release. Cytokine release correlated with nuclear PPARγ localization; CSE reduced the amount of activated PPARγ located in the nucleus and formed aldehyde adducts as PPARγ protein carbonyls. Furthermore, it was shown that PPARγ interacts with the RelA/p65 subunit of NF-κB under TNF-α exposure conditions, but this interaction was disrupted by CS exposure, suggesting that CS blocks this important anti-inflammatory pathway involving PPARγ. Thus, these new data show that activation of PPARγ with natural or synthetic ligands have differential inhibitory effects on CS-mediated pro-inflammatory mediator release. These data have implications in designing therapies for treatment of COPD and pulmonary fibrosis. *Antioxid. Redox Signal.* 10, 253–260.

INTRODUCTION

Peroxisome Proliferator-Activated Receptor gamma (PPARγ) is a member of the ligand-activated nuclear receptor superfamily. PPARγ is located in the cytoplasm under basal conditions; however, when bound to ligand, it is translocated into the nucleus and regulates several metabolic pathways by binding to sequence-specific PPAR response elements in the promoter region of target genes (2, 5, 36). Studies have shown that endogenous 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), which is generated through the cyclooxygenase-2 (COX-2) pathway, blocks inflammation (3, 20) and, being an electrophilic compound, can also induce phase II antioxidant genes through the nuclear factor E2-related factor 2 (Nrf2) mediated pathway (13). In addition to natural ligands, a wide range of synthetic PPARγ agonists such as rosiglitazone, ciglitazone, and pioglitazone have been produced. Synthetic PPARγ agonists are well-known "insulin sensitizers" and are used in the treatment of type II diabetes. Amongst those, rosiglitazone is the most potent and selective agent which binds PPARγ with high affinity. PPARγ also has the ability to physically interact with NF-κB, resulting in trans-repression of NF-κB signaling, which could contribute to the anti-inflammatory effects of PPARγ agonists (36). Recently PPARγ ligands have also been

Department of Environmental Medicine, Lung Biology and Disease Program, University of Rochester Medical Center, Rochester, New York.

proposed as anti-inflammatory agents in the treatment of a wide range of inflammatory diseases, including chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (5, 6, 31).

Cigarette smoke (CS)-triggered inflammation plays a central role in the development of COPD by a mechanism that involves enhanced pro-inflammatory gene transcription (26). In addition, it has been observed in heavy smokers that both emphysema and pulmonary fibrosis coexist, with fibrotic lesions in the lower zones/parenchyma and emphysema present in the upper zones of the lung (11). Moreover, exposure to CS has also been shown to associate with the development of several interstitial lung diseases and pulmonary fibrosis (11). It is therefore critical to understand the role of CS-induced lung inflammation in the development of these chronic inflammatory lung diseases. Cigarette smoke contains an estimated 10^{15-17} oxidants/free radicals and ~4,700 different chemical compounds per puff, including reactive aldehydes and quinones (9). Macrophages are proposed as a key inflammatory cell which orchestrates the inflammatory response in the lungs of COPD patients (29). Recently, our laboratory demonstrated that CS extract (CSE) activates NF-κB and pro-inflammatory cytokine release in macrophages and rat lungs (19, 32). In addition, IκB-kinase β (IKKβ) and/or MAP kinase inhibitors were unable to completely block the CSE-induced cytokine release from macrophages (32), suggesting the involvement of an alternative pathway in CS-mediated inflammation. This is supported by the fact that current therapies for COPD are not effective (1, 5, 26, 31). For example, corticosteroid treatment, which alleviates inflammation in asthma, is ineffective in COPD (1). Similarly, IKK2 (NF-κB) inhibitors are ineffective and have immunosuppressive side effects (26). Since corticosteroids and IKK2 inhibitors are not effective in controlling CS-induced lung inflammation, it was hypothesized that PPARγ agonists, that possess anti-inflammatory activity, would inhibit CS-induced pro-inflammatory cytokine release in macrophages. To test this hypothesis, the effect of selected synthetic (rosiglitazone) and natural (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) PPARγ agonists on CS and TNF-α-induced pro-inflammatory cytokine release (IL-8) was investigated in the monocyte–macrophage MonoMac6 cell line. The interactions of PPARγ and the RelA/p65 subunit of NF-κB in response to PPARγ ligands was also studied to determine the mechanism of anti-inflammatory effect of certain PPARγ agonists in CS-induced pro-inflammatory cytokine release in MonoMac6 cells.

MATERIALS AND METHODS

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Chemicals Co., St. Louis, MO.

Materials

Penicillin, streptomycin, and RPMI 1640 were obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT). Rosiglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 were obtained from Cayman (Ann Arbor, MI). Mouse monoclonal antibodies to PPARγ (sc-7273), mouse monoclonal antibodies to α-tubulin (sc-5286), rabbit polyclonal antibodies to NF-κB RelA/p65 (sc-372), and goat polyclonal antibodies to Lamin B (sc-6216) were purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Rabbit polyclonal antibodies to PPAR (SA-206) were also purchased from Biomol International (Plymouth Meeting, PA), and mouse monoclonal antibodies to 4-hydroxy-2-nonenal (4-HNE) were purchased from Oxis International (Foster City, CA).

MonoMac6 cell culture

The human monocyte–macrophage cell line (mature monocyte–macrophage) MonoMac6, which was established from peripheral blood of a patient with monoblastic leukemia (21, 35), were grown in RPMI 1640 medium supplemented with 10% FBS, 2 m*M* L-glutamine, 100 μg/ml penicillin, 100 unit/ml streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, 1 μg/ml human holo-transferrin, 1 m*M* oxaloacetic acid, and 9 μg/ml bovine insulin. These cells do not require phorbol 12-myristate 13-acetate (PMA) or 1,2,5-dihydroxyvitamin D_3 to differentiate into macrophages, thus avoiding any stress to the cells. The cells were cultured at 37°C in a humidified atmosphere containing 7.5% CO_2.

Preparation of aqueous cigarette smoke extract

Research grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky, Lexington, KY. The total particulate matter (TPM) content of 1R3F was 17.1 mg/cigarette, tar (15 mg/cigarette) and nicotine (1.16 mg/cigarette). 10% CSE was prepared by bubbling smoke from one cigarette into 10 ml of culture media supplemented with 1% FBS at a rate of one cigarette/2 min, as described previously (16, 22, 32), using a modification of the method described by Carp and Janoff (7). The pH of the CSE was adjusted to 7.4 and was sterile filtered through a 0.45 μm filter (25 mm Acrodisc; Pall Corporation, Ann Arbor, MI). The CSE preparation was standardized by monitoring the absorbance at 320 nm (OD 0.74 $\pm$ 0.05). The spectral variations observed between different CSE preparations at λ320 were minimal. CSE was freshly prepared for each experiment and diluted with culture media containing 1% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml of culture media supplemented with 1% FBS, adjusting pH adjusted to 7.4, and sterile filtered as described for 10% CSE.

Treatments

MonoMac6 cells were seeded at a density of 4.5×10^6 cells/well (final volume 10 ml) and grown to ~80–90% confluency in 100 mm dishes containing RPMI media with 1% FBS and treatment media contained 1% serum as well. All treatments were performed in duplicate. The cells were treated with CSE (0.5%) or TNF-α (10 ng/ml) for 4 h in the presence or absence of a 2 h pretreatment with rosiglitazone (10 μ*M*), or for 24 h with CSE (1% and 5%) or TNF-α (10 ng/ml) with a cotreatment of 15d-PGJ_2 (1 and 5 μ*M*) at 37°C with 7.5% CO_2.

At the end of treatment, the cells were washed with cold sterile Ca^{2+}/Mg^{2+}-free PBS and were lysed before being used to assay PPARγ and its interaction with RelA/p65. Culture media from these cells were collected and stored at −80°C until analyzed for IL-8 release.

Western blot analysis

MonoMac6 cells were exposed to CSE or TNF-α, with or without PPARγ ligands, as described above. Nuclear proteins were extracted from MonoMac6 cells with 10% Nonidet P-40 (NP-40) lysis buffer supplemented with a protease inhibitor cocktail [leupeptin, aprotinin, pepstatin, and phenylmethylsulphonylfluoride (PMSF)]. For PPARγ assays, 20 μg of isolated nuclear proteins were subjected to electrophoresis on 7.5% PAGE gels and transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL) using an electro-blotting technique. The nitrocellulose membrane was blocked with 10% nonfat milk and subsequently incubated with mouse polyclonal anti-human PPARγ antibody (1:1000 dilution, Santa Cruz). After three washing steps (10 min each), the levels of protein were detected using goat anti-mouse antibody (1:20,000 dilution) linked to horseradish peroxidase (Dako, Santa Barbara, CA), and bound complexes were detected using enhanced chemiluminescence method (ECL, Jackson Immunology Research, West Grove, PA).

RelA/p65 immunoprecipitation and its interaction with PPARγ

MonoMac6 cells were treated with CSE (0.5%) or TNF-α (10 ng/ml), with or without PPARγ ligands for 4 h at 37°C. The nuclear fraction was then isolated and RelA/p65 immunoprecipitated: RelA/p65 antibody (1:80 dilution, Santa Cruz) was added to 100 μg of protein in a final volume of 400 μl and incubated for 1 h. Protein-A/G agarose beads (20 μl; Santa Cruz) were added to each sample and incubated overnight at 4°C on a rotator. The samples were then centrifuged at 13,000 rpm at 4°C for 5 min. The supernatant was discarded and the beads were washed three times and then resuspended in 50 μl lysis buffer. For Western blots, 100 μg of the immunoprecipitated RelA/p65 agarose bead suspension was added to 20 μl of 5× sample buffer, boiled, and resolved using SDS-PAGE as described above. Agarose beads alone were used as negative control. To demonstrate the interaction of PPARγ with RelA/p65 subunit of NF-κB, immunoprecipitated RelA/p65 was blotted against PPARγ.

Post-translational modification of PPARγ

MonoMac6 cells were treated with CSE for 4 h at 37°C, nuclear fraction was isolated, and PPARγ was immunoprecipitated: PPARγ antibody (1:80 dilution, Biomol) was added to 100 μg of protein in a final volume of 400 μl and incubated for 1 h. Protein-A/G agarose beads (20 μl; Santa Cruz) were added to each sample and left overnight at 4°C on a rotator. The samples were then centrifuged at 13,000 rpm at 4°C for 5 min. The supernatant was discarded and the beads were washed three times and then resuspended in 50 μl lysis buffer. For Western blots, 100 μg of the immunoprecipitated PPARγ agarose bead suspension was added to 20 μl of 5× sample buffer, boiled, and resolved using SDS-PAGE as described above. Agarose beads alone were used as negative control. To determine the post-translational modification of PPARγ, blots were probed with mouse monoclonal anti-4-hydroxy-2-nonenal (4-HNE) antibody (Oxis International) and followed by anti-PPARγ antibody (Biomol).

Enzyme-linked immunosorbant assay (ELISA) for IL-8

The culture medium was collected following treatment and centrifuged at 2,500 for 5 min to pellet the cells. The supernatant was then removed and stored at −80°C prior to analysis. The levels of IL-8 in the supernatants were determined by ELISA using the duo antibody kit from R&D Systems Inc. (Minneapolis, MN) according to the manufacture's instructions.

Protein assay

Protein levels were measured using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). Protein standards were obtained by diluting a stock solution of bovine serum albumin (BSA). Linear regression was used to determine the actual protein concentration of the samples.

Statistical analysis

The results are shown as the mean ± SEM of four to six experiments. Statistical analysis of significance was calculated using one-way Analysis of Variance (ANOVA) using STATVIEW; p values > 0.05 considered nonsignificant, and */† $p < 0.05$; **/†† $p < 0.01$; and ***/††† $p < 0.001$ as significant.

RESULTS

Cigarette smoke induces IL-8 release in MonoMac6 cells at 4 h

The earliest time at which MonoMac6 cells would release IL-8, an NF-κB-dependent pro-inflammatory cytokine, in response to CSE, was determined. There was no differential IL-8 release prior to 2 h (data not shown). However, at 4 h, MonoMac6 cells had significantly increased IL-8 release by 280% above control in response to 0.5% CSE ($p < 0.001$) (Fig. 1). MonoMac6 cells were then treated for the remainder of this study using 4 h as a standard time point for cigarette smoke-mediated IL-8 release.

15d-PGJ_2 enhances both TNF-α- and cigarette smoke extract-mediated IL-8 release

The effects of activating PPARγ with the endogenous ligand 15d-PGJ_2 on IL-8 release were next examined. MonoMac6 cells were co-treated with 15d-PGJ_2 and either TNF-α or CSE for 24 h, as there was no effect of 15d-PGJ_2 observed at earlier time points. Alone, 15d-PGJ_2 significantly increased IL-8 release above untreated control cells by 278% ($p < 0.001$) at both 1 and 5 μM (Fig. 2). Surprisingly, IL-8 levels were also sig-

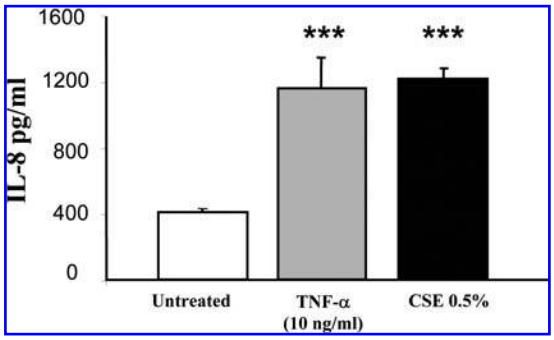

FIG. 1. Cigarette smoke extract (CSE) induces IL-8 release from MonoMac6 cells at 4 h. MonoMac6 cells were treated for 4 h with freshly prepared CSE (0.5%) or TNF-α (10 ng/ml) as a positive control. Cells were harvested, and supernatants were collected for measuring IL-8 levels by ELISA. 0.5% CSE showed increased levels of IL-8. Each histogram represents the mean of four experiments; $***p < 0.001$ compared to control values.

nificantly higher when MonoMac6 cells were treated in combination with TNF-α and 15d-PGJ_2, and CSE (1 and 5%) and 15d-PGJ_2. These data suggest pro-inflammatory, rather than anti-inflammatory, effects of 15d-PGJ_2 on MonoMac6 cells.

Activation of PPARγ with rosiglitazone downregulates TNF-α-, but not cigarette smoke extract, mediated IL-8 release

To determine the anti-inflammatory effects of synthetic PPARγ ligand rosiglitazone on IL-8 release in MonoMac6 cells, the cells were pretreated with rosiglitazone for 2 h, followed by treatment with either TNF-α or CSE. Rosiglitazone treatment decreased TNF-α mediated IL-8 release by 57%, however, it had no effect on CSE mediated IL-8 release (Fig. 3). This observation suggested that CSE-derived free radicals may interfere in PPARγ agonist mediated anti-inflammatory effects in macrophages.

Cigarette smoke extract decreased the levels of nuclear PPARγ

It was next evaluated whether the reduction in IL-8 release by rosiglitazone was due to PPARγ translocating to the nucleus and whether this translocation was impaired by CSE. Treatment with rosiglitazone alone showed increased PPARγ levels in the nucleus (Fig. 4). Increased nuclear PPARγ levels were also associated with cells treated with rosiglitazone plus TNF-α. Cells treated only with CSE treatment had low levels of PPARγ in the nucleus, and pretreatment with rosiglitazone did not further increase nuclear PPARγ levels compared to control value (100%). These data suggest that CSE interferes with PPARγ signaling in macrophages.

PPARγ possesses an active sulfhydryl group in its active site pocket (16). This sulfhydryl group is subject to modifications by reactive aldehydes and free radicals present in CS. In order to determine whether PPARγ is modified post-translationally by reactive aldehydes present in CS, PPARγ from MonoMac6 cells treated with CSE was immunoprecipitated and immunoblotted for 4-hydroxy-2-nonenol (4-HNE) (Fig. 5). CSE treatment increased the formation of 4-HNE-protein carbonyl adducts with PPARγ, suggesting that PPARγ was modified post-translationally by CS-derived aldehydes, and this modification may be the reason for inefficacy of PPARγ in ameliorating the CS-induced release of IL-8 from macrophages.

Cigarette smoke extract disrupts the association between PPARγ and RelA/p65 of NF-κB

PPARγ interacts with RelA/p65, and this is postulated to be responsible for its anti-inflammatory effects (36). In light of our data showing no inhibitory effect of PPARγ ligands

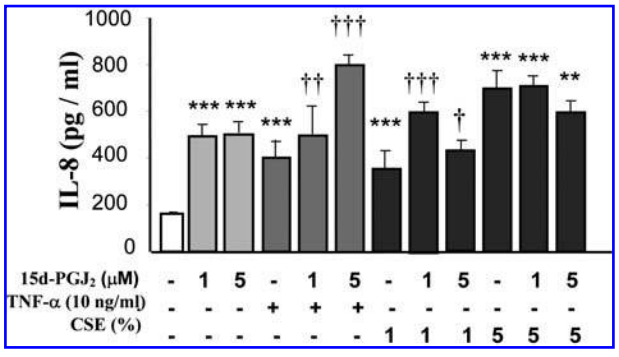

FIG. 2. Exposure to 15d-PGJ_2 enhanced both TNF-α and cigarette smoke extract (CSE) mediated IL-8 release in MonoMac6 cells. MonoMac6 cells were co-treated with 15-deoxy-PGJ_2 (1 and 5 μM) and either TNF-α (10 ng/ml) or CSE (1 and 5%) for 24 h, and cell culture media was used for IL-8 analysis by ELISA. The results are presented as an average of six separate experiments performed in duplicate; $***p < 0.001$ compared to control values and $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ and $\dagger\dagger\dagger p < 0.001$ compared to relevant treatment.

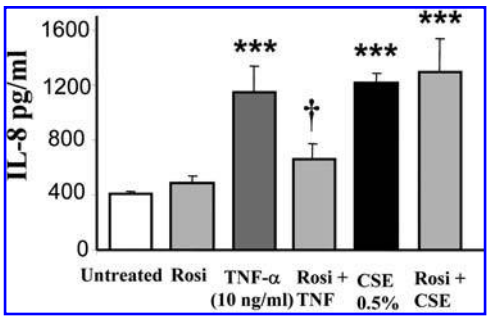

FIG. 3. Exposure to rosiglitazone downregulates TNF-α, but not cigarette smoke extract (CSE), mediated IL-8 release in MonoMac6 cells. MonoMac6 cells were pretreated with rosiglitazone (10 μM) for 2 h, and then incubated with freshly prepared CSE (0.5%) or TNF-α (10 ng/ml) for 4 h. IL-8 release was measured by ELISA. The results are presented as an average of four separate experiments performed in duplicate; $***p < 0.001$ compared to control values and $\dagger p < 0.5$ compared to TNF-α.

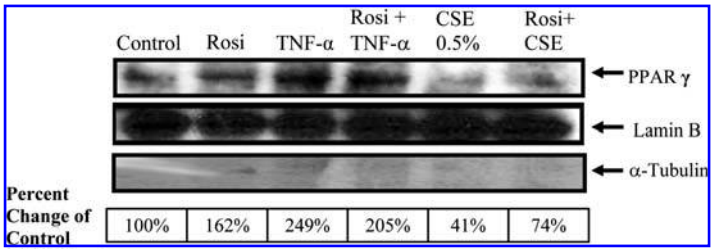

FIG. 4. Cigarette smoke extract (CSE) decreased the levels of nuclear PPARγ protein at 4 h in MonoMac6 cells pretreated with rosiglitazone. MonoMac6 cells were pretreated with rosiglitazone (10 μM) for 2 h, and then incubated with freshly prepared CSE (0.5%) or TNF-α (10 ng/ml) for 4 h. Western blots of soluble nuclear proteins (20 μg) extracted from treated MonoMac6 cells were electrophoresed on 7.5% PAGE gels and electroblotted onto nitrocellulose membranes using anti-PPARγ antibody. PPARγ band size was the same as predicted (68 *k*Da). Lamin B was used as both a loading control and for determining nuclear purity. Lamin B band size was the same as predicted (67 *k*Da). α-Tubulin was used to show lack of cytoplasmic proteins in the nuclear extracts. α-Tubulin band size was the same as predicted (55 *k*Da). Data are shown as % change versus control value set at 100%.

on CSE-induced IL-8 release and low levels of nuclear PPARγ in MonoMac6 cells, it was hypothesized that CSE, which activates NF-κB (32), would disrupt PPARγ-RelA/p65 interactions due to low levels of nuclear PPARγ in MonoMac6 cells. This hypothesis was tested by determining the possible interactions between PPARγ and RelA/p65 subunit of NF-κB in the nucleus in response to rosiglitazone and TNF-α and CSE treatment. RelA/p65 was immunoprecipitated from nuclear extracts of MonoMac6 cells and Western blotting was performed for PPARγ. There was increased association between PPARγ and RelA/p65 in cells treated with both TNF-α and rosiglitazone, compared to cells treated with TNF-α alone (Fig. 6). In contrast, there was no association between RelA/p65 and PPARγ in cells treated with both rosiglitazone and CSE; suggesting that this association was disrupted by CSE. These results suggested that the association between RelA/p65 and PPARγ is important for lowering cytokine release, as reduction in IL-8 was only seen in rosiglitazone and TNF-α treated cells; suggesting that CS disrupts this essential interaction.

DISCUSSION

Alveolar macrophages are important cells in perpetuating the inflammatory response to CS (29), particularly by their release of pro-inflammatory mediators. It has been shown previously that CS induces pro-inflammatory cytokine release via activation of NF-κB in monocyte–macrophage cell line (MonoMac6) (32). Herein, IL-8 release from MonoMac6 cells was continued to be examined, finding that the earliest these cells differentially respond to increasing doses of CSE was at 4 h, and that a dose of cigarette smoke as low as 0.5% could induce IL-8 release. Using 4 h as a time point for early events induced by smoke, MonoMac6 cells were treated with two types of PPARγ agonists to determine if these agents could decrease CSE-induced IL-8 release. Several studies have suggested that PPARγ agonists are potential therapeutic agents for patients suffering from COPD for whom glucocorticoid therapy is ineffective (4, 5, 25, 31). However, this is the first report of the effect of PPARγ agonists on macrophages in response to CSE-induced cytokine release. We focused our study on two types of PPARγ agonists; the most potent thiazolidinedione, rosiglitazone, and a natural agonist, 15d-PGJ_2.

15d-PGJ_2 is a natural product of arachidonic acid metabolism, metabolized from prostaglandin D_2, and a known endogenous PPARγ ligand. Since 15d-PGJ_2 is generated during inflammation (30) and can inhibit macrophage functions (2, 3), we determined whether 15d-PGJ_2 could decrease CSE induced cytokine release in MonoMac6 cells. Surprisingly, when the cells were treated with 15d-PGJ_2 alone, IL-8 release was increased, rather than decreased, compared to the basal levels. Furthermore, when the cells were treated in combination with either CSE or TNF-α, 15d-PGJ_2 augmented IL-8 release. Our data corroborate the recent findings that 15d-PGJ_2 can increase IL-8 expression in THP-1 macrophages (10), and when given in combination with lipopolysaccharide (LPS), 15d-PGJ_2 further increased IL-8 expression in human monocytes (34). 15d-PGJ_2, being an electrophilic molecule, may be increasing IL-8 release in MonoMac6 cells through a PPARγ-independent mechanism. It has been shown that in blood macrophages 15d-PGJ_2 can exert both anti-inflammatory (12) and pro-inflammatory effects by inducing IL-8 synthesis (14) without binding to PPARγ. Furthermore, it is known that certain PPARγ-independent pro-inflammatory effects of 15d-PGJ_2 are mediated by a ROS-dependent mechanism (17, 27). It remains to be determined whether 15d-PGJ_2 can cause ROS production in MonoMac6 cells. However, our preliminary data (not shown) revealed that 15d-PGJ_2 can in fact trigger both cytosolic (increased DCF-DA staining) and mitochondrial (increased Mitosox Red staining) ROS release in MonoMac6 cells.

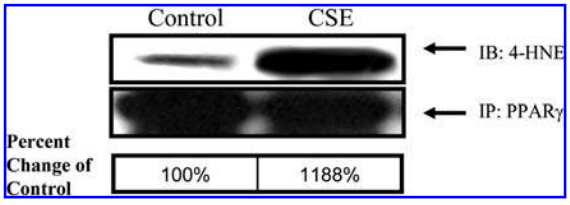

FIG. 5. Cigarette smoke extract (CSE) causes post-translational modification of PPARγ. 4-hydroxy-2-nonenol (4-HNE) modification of PPAR is increased after CSE treatment in MonoMac6 cells compared with control. PPARγ was immunoprecipitated (IP) from MonoMac6 cells treated for 4 h with CSE (5%). Equal amount (100 μg) of immunoprecipitated PPARγ protein was used for Western blots. Levels of 4-HNE were analyzed by immunobloting (IB). Data are shown as % change versus control value set at 100%.

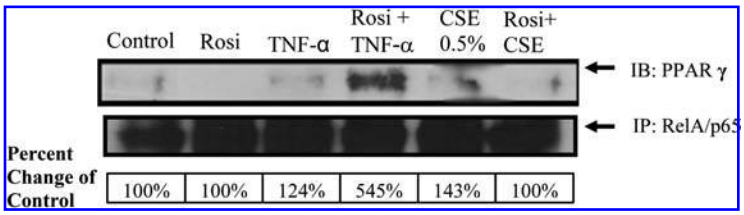

FIG. 6. TNF-α, but not cigarette smoke extract (CSE), induced IL-8 release was associated with increased interactions between PPARγ and RelA/p65 subunit of NF-κB. RelA/p65 was immunoprecipitated (IP) from MonoMac6 cells pretreated for 2 h with rosiglitazone (10 μM), followed by CSE (0.5%) or TNF-α (10 ng/ml) treatment for 4 h. Equal amount (100μg) of immunoprecipitated RelA/p65 protein was used for Western blots. Levels of PPARγ were analyzed by immunobloting (IB). Pretreatment with rosiglitazone increased nuclear localization of a PPARγ-RelA/p65 complex in TNF-α, but not CSE (0.5%) treated MonoMac6 cells. Data are shown as % change versus control value set at 100%.

The effects of rosiglitazone on cytokine release from MonoMac6 cells were then examined. Thiazolidinediones can decrease LPS-induced cytokine release from human alveolar macrophages (2) and also decrease CS-induced mucin secretion from human airway epithelial (NCI-H292) cells (18). Therefore, it seemed possible that these compounds would inhibit CSE-induced cytokine release. However, our data show that rosiglitazone could only reduce TNF-α-mediated IL-8 release, but not CSE-induced IL-8 release, in MonoMac6 cells. Treatment of cells with rosiglitazone alone increased nuclear PPARγ levels, as did treatment with both rosiglitzaone and TNF-α, suggesting that the decrease in IL-8 release was due to a PPARγ-dependent mechanism. Whether PPARγ ligands use PPARγ-independent and/or -dependent mechanisms to exert anti-inflammatory effects remains the subject of much debate (8). It has been observed that PPARγ ligands do not inhibit basal TNF-α release from THP-1 macrophages (23). However, our result showing rosiglitazone-mediated reduction in IL-8 release from MonoMac6 cells was contradictory to the above finding possibly due to the use of different cell lines (PMA activated THP-1 cells vs. non-PMA treated MonoMac6 cells). Furthermore, we observed lower levels of PPARγ protein in the nucleus of cells exposed to CS with or without rosiglitazone treatment. This suggests that CS interferes with PPARγ signaling due to direct binding of reactive aldehydes present in CSE with cysteine residue in the active site of PPARγ binding pocket (15) and thus retaining PPARγ in the cytoplasm. Indeed, our results show that PPARγ was modified by 4-HNE forming carbonyl adducts.

It was also investigated whether rosiglitazone-activated PPARγ had any effects on NF-κB signaling. Ligand-activated PPARγ can play a negative regulatory role in macrophage activation, resulting in less NF-κB activity (28), and that PPARγ and the RelA/p65 subunit of NF-κB can interact, dampening pro-inflammatory signals (36). Our data show that when cells were treated with rosiglitazone plus TNF-α, PPARγ and RelA/p65 associated together in the nucleus. However, there was less association between the two nuclear proteins when cells were treated with CSE, with or without rosiglitazone. This observation suggests that when macrophages were treated with rosiglitazone, PPARγ translocated to the nucleus and interacted with NF-κB to decrease IL-8 release. Cigarette smoke disrupts this complex and diminishes PPARγ's anti-inflammatory capabilities. The mechanism for CS-mediated disruption of this complex is not known, but our data suggests that CS-derived reactive aldehydes interact with the cysteine pocket in PPARγ (15), thereby forming protein carbonyl adducts and interfering in the protein-protein interactions between PPARγ and RelA/p65. An alternative explanation of this protein-protein disruption comes from the observation that CSE activates RelA/p65 subunit of NF-κB by phosphoacetylation of the lysine 310 residue, which can not be reversed by IκBα (33), and this phosphoacteylation impairs the interactions between RelA/p65 and agonist-activated PPARγ. Our finding of CS-mediated interference of PPARγ action is interesting in light of recent research showing that PPARγ can also interact with the glucocorticoid receptor (24), whose signaling is also disrupted by smoke (1).

Overall, our data show that two PPARγ ligands have very different effects (Fig. 7). Although a naturally occurring compound, 15d-PGJ$_2$ increased both TNF-α and CSE-induced IL-8 release, whereas the synthetic rosiglitazone could only decrease TNF-α-, but not CSE-, induced cytokine release. 15d-PGJ$_2$ can increase cellular ROS (27) which could further drive inflammation, while rosiglitazone bound to PPARγ allowed for complex formation with RelA/p65 to decrease pro-inflammatory cytokine release. Our study provides insight into a class of compounds that are proposed to have the potential to help patients with COPD and fibrosis/interstitial lung diseases. However, our data suggest that for chronic smokers and for patients with COPD, PPARγ agonists may not decrease CS-induced release of pro-inflammatory mediators. Additionally, it

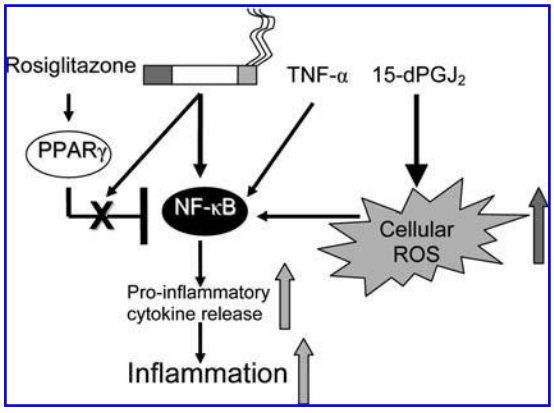

FIG. 7. Model for PPARγ-dependent and -independent mechanism of action of PPARγ ligands. Rosiglitazone-activated PPARγ forms a complex with the RelA/p65 subunit of NF-κB in response to TNF-α treatment, decreasing NF-κB dependent gene transcription. Cigarette smoke extract (CSE) disrupts this complex allowing for pro-inflammatory gene transcription. 15d-PGJ$_2$ works through a PPARγ-independent mechanism to increase ROS and pro-inflammatory mediators.

has been shown that PPARγ agonists inhibit TGF-α-induced pulmonary myofibroblast differentiation and collagen production (6). However, these agonists may not decrease the underlying CS-induced inflammation which is seen in patients with COPD, pulmonary fibrosis, and other interstitial lung diseases. Further work is required to confirm these *in vitro* findings using preclinical animal models of CS exposure and primary monocytes/macrophages obtained from patients with COPD, pulmonary fibrosis, and various interstitial lung diseases.

ACKNOWLEDGMENTS

This study was supported by the NIEHS Environmental Health Sciences Center (ES01247), NIEHS Toxicology Training Program Grant (ES07026), and by HL085613, DE011390, HL078603, HL086367, and EY017123.

ABBREVIATIONS

COPD, chronic obstructive pulmonary disease; COX-2, cyclooxygenase 2; CS, cigarette smoke; CSE, cigarette smoke extract; 15d-PGJ_2,15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2; FBS, fetal bovine serum; 4-HNE, 4-hydroxy-2-nonenal; IκBα, inhibitor of kappaB α; IKKβ, IκB-kinase β; IL-8, interleukin 8; LPS, lipopolysaccharide; PMSF, phenylmethylsulphonylfluoride; PPARγ, peroxisome proliferator-activated receptor gamma;NF-κB, nuclear factor-kappaB; Nrf2, nuclear factor E2 related factor 2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha;TGF-β, transforming growth factor-beta.

REFERENCES

1. Adcock IM and Ito K. Glucocorticoid pathways in chronic obstructive pulmonary disease therapy. *Proc Am Thorac Soc* 2: 340–341, 2005.
2. Asada K, Sasaki S, Suda T, Chida K, and Nakamura H. Antiinflammatory roles of peroxisome proliferator-activated receptor gamma in human alveolar macrophages. *Am J Resp Crit Care Med* 169: 145–146, 2004.
3. Azuma Y, Shinohara M, Wang PL, and Ohura K. 15-Deoxy-delta(12,14)-prostaglandin J_2 is a negative regulator of macrophage functions. *Int Immunopharmacol* 1: 2101–2108, 2001.
4. Becker J, Delayre–Orthez C, Frossard N, and Pons F. Regulation of inflammation by PPARs: a future approach to treat lung inflammatory diseases? *Fundam Clin Pharmacol* 20: 429–447, 2006.
5. Belvisi MG, Hele DJ, and Birrell MA. Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. *Eur J Pharmacol* 533: 101–109, 2006.
6. Burgess HA, Daugherty LE, Thatcher TH, Lakatos HF, Ray DM, Reddonet M, Phipps RP, and Sime PJ. PPARgamma agonists inhibit TGF-beta induced pulmonary myofibroblast differentiation and collagen production: implications for therapy of lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 288: L1146–11453, 2005.
7. Carp H and Janoff A. Possible mechanisms of emphysema in smokers. In vitro suppression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *Am Rev Respir Dis* 118: 617-621, 1978.
8. Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, and Evans RM. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med* 7: 48–52, 2001.
9. Church DF and Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 64: 111–126, 1985.
10. Fu Y, Luo N, and Lopes–Virella MF. Upregulation of interleukin-8 expression by prostaglandin D2 metabolite 15-deoxy-delta12, 14 prostaglandin J_2 (15d-PGJ_2) in human THP-1 macrophages. *Atherosclerosis* 160: 11–20, 2002.
11. Gauldie J, Kolb M, Ask K, Martin G, Bonniaud P, and Warburton D. Smad3 signaling involved in pulmonary fibrosis and emphysema. *Proc Am Thorac Soc* 3: 696–702, 2006.
12. Hinz B, Brune K, and Pahl A. 15-Deoxy-delta(12,14)-prostaglandin J_2 inhibits the expression of proinflammatory genes in human blood monocytes via a PPAR-gamma-independent mechanism. *Biochem Biophys Res Commun* 302: 415–420, 2003.
13. Itoh K, Mochizuki M, Ishii Y, Ishii T, Shibata T, Kawamoto Y, Kelly V, Sekizawa K, Uchida K, and Yamamoto M. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-delta(12,14)-prostaglandin J(2). *Mol Cell Biol* 24: 36–45, 2004.
14. Jozkowicz A, Dulak J, Prager M, Nanobashvili J, Nigisch A, Winter B, Weigel G, and Huk I. Prostaglandin-J2 induces synthesis of interleukin-8 by endothelial cells in a PPAR-gamma-independent manner. *Prostaglandins Other Lipid Mediat* 66: 165–177, 2001.
15. Kim EH and Surh YJ. 15-deoxy-delta-12,14-prostaglandin J_2 as a potential endogenous regulator of redox-sensitive transcription factors. *Biochem Pharmacol* 72: 1516–1528, 2006.
16. Kode A, Yang SR, and Rahman I. Cigarette smoke induces proinflammatory cytokine release in primary airway epithelial cells but not in transformed alveolar epithelial cells. *Respir Res* 7: 132, 2006.
17. Landar A, Zmijewski JW, Dickinson DA, Le Goffe C, Johnson MS, Milne GL, Zanoni G, Vidari G, Morrow JD, and Darley–Usmar VM. Interaction of electrophilic lipid oxidation products with mitochondria in endothelial cells and formation of reactive oxygen species. *Am J Physiol Heart Cric Physiol* 290: H1777–1787, 2006.
18. Lee SY, Kang EJ, Hur GY, Jung KH, Jung HC, Lee SY, Kim JH, Shin C, In KH, Yoo SH, and Shim JJ. Peroxisome proliferators-activated receptor-gamma inhibits cigarette smoke solution-induced mucin production in human airway epithelial (NCI-H292) cells. *Am J Physiol Lung Cell Mol Physiol* 291: L84–90, 2006.
19. Marwick JA, Kirkham PA, Stevenson CS, Danahay H, Giddings J, Butler K, Donaldson K, Macnee W, and Rahman I. Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs. *Am J Respir Cell Mol Biol* 31: 633–642, 2004.
20. Mochizuki M, Ishii Y, Itoh K, Iizuka T, Morishima Y, Kimura T, Kiwamoto T, Matsuno Y, Hegab AE, Nomura A, Sakamoto T, Uchida K, Yamamoto M, and Sekizawa K. Role of 15-deoxy delta(12,14) prostaglandin J_2 and Nrf2 pathways in protection against acute lung injury. *Am J Respir Crit Care Med* 171: 1260–1266, 2005.
21. Moesby L, Jensen S, Hansen EW, and Christensen JD. A comparative study of Mono Mac 6 cells, isolated mononuclear cells and Limulus amoebocyte lysate assay in pyrogen testing. *Int J Pharm* 191: 141–149, 1999.
22. Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, Kilty I, and Rahman I. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. *FASEB J* 18: 1897–1899, 2004.
23. Naitoh T, Kitahara M, and Tsuruzoe N. The effect of peroxisome proliferator-activated receptor gamma (PPARgamma) on human monocyte function: PPARgamma ligands do not inhibit tumor necrosis factor-alpha release in human monocytic cell line THP-1. *Cell Biol Toxicol* 16: 131–135, 2000.
24. Nie M, Corbett L, Knox AJ, and Pang L. Differential regulation of chemokine expression by peroxisome proliferator-activated receptor gamma agonists: interactions with glucocorticoids and beta2-agonists. *J Biol Chem* 280: 2550–2561, 2005.
25. Patel HJ, Belvisi MG, Bishop–Bailey D, Yacoub MH, and Mitchell JA. Activation of peroxisome proliferator-activated receptors in human airway smooth muscle cells has a superior anti-inflammatory profile to corticosteroids: relevance for chronic obstructive pulmonary disease therapy. *J Immunol* 170: 2663–2669, 2003.

26. Rahman I and Adcock IM. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur Resp J* 28: 219–242, 2006.
27. Ray DM, Akbiyik F, and Phipps RP. The peroxisome proliferator-activated receptor gamma (PPARgamma) ligands 15-deoxy-delta12,14-prostaglandin J_2 and ciglitazone induce human B lymphocyte and B cell lymphoma apoptosis by PPARgamma-independent mechanisms. *J Immunol* 177: 5068–5076, 2007.
28. Ricote M, Li AC, Willson TM, Kelly CJ, and Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391: 79–82, 1998.
29. Shapiro SD. The macrophage in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 160: S29–32, 1999.
30. Shibata T, Kondo M, Osawa T, Shibata N, Kobayashi M, and Uchida K. 15-deoxy-delta 12,14-prostaglandin J_2. A prostaglandin D2 metabolite generated during inflammatory processes. *J Biol Chem* 277: 10459–10466, 2002.
31. Spears M, McSharry C, and Thomson NC. Peroxisome proliferator-activated receptor-gamma agonists as potential anti-inflammatory agents in asthma and chronic obstructive pulmonary disease. *Clin Exp Allergy* 36: 1494–1504, 2006.
32. Yang SR, Chida AS, Bauter MR, Shafiq N, Seweryniak K, Maggirwar SB, Kilty I, and Rahman I. Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and post-translational modifications of histone deacetylase in macrophages. *Am J Physiol Lung Cell Mol Physiol* 291: L46–57, 2006.
33. Yang SR, Wright J, Bauter M, Seweryniak K, Kode A, and Rahman I. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. *Am J Physiol Lung Cell Mol Physiol* 292: L567–576, 2007.
34. Zhang X, Wang JM, Gong WH, Mukaida N, and Young HA. Differential regulation of chemokine gene expression by 15-deoxy-delta 12, 14 prostaglandin J_2. *J Immunol* 166: 7104–7111, 2001.
35. Ziegler–Heitbrock HW, Thiel E, Futterer A, Herzog V, Wirtz A, and Riethmuller G. Establishment of a human cell line (Mono-Mac6) with characteristics of mature monocytes. *Int J Cancer* 41: 456–461, 1988.
36. Zingarelli B and Cook JA. Peroxisome proliferator-activated receptor-γ is a new therapeutic target in sepsis and inflammation. *Shock* 23: 393–399, 2005.

Address reprint requests to:
Dr. Irfan Rahman
Department of Environmental Medicine
Division of Lung Biology and Disease
University of Rochester Medical Center
601 Elmwood Ave., Box 850
Rochester, NY 14642

E-mail: irfan_rahman@urmc.rochester.edu

Date of first submission to ARS Central, August 28, 2007; date of acceptance, September 4, 2007.

This article has been cited by:

1. Paul A. Kirkham, Gaetano Caramori, K. Fan Chung, Ian M. AdcockNovel Anti-Inflammatory Treatments for Asthma and COPD 154-202. [CrossRef]
2. Hongwei Yao, Irfan RahmanEnvironment and the Role of Inflammation in Chronic Pulmonary Diseases **20115234**, . [CrossRef]
3. A Amoruso, G Gunella, E Rondano, C Bardelli, LG Fresu, V Ferrero, F Ribichini, C Vassanelli, S Brunelleschi. 2009. Tobacco smoke affects expression of peroxisome proliferator-activated receptor-# in monocyte/macrophages of patients with coronary heart disease. *British Journal of Pharmacology* **158**:5, 1276-1284. [CrossRef]
4. Maria G Belvisi, Jane A Mitchell. 2009. Targeting PPAR receptors in the airway for the treatment of inflammatory lung disease. *British Journal of Pharmacology* **158**:4, 994-1003. [CrossRef]
5. Vuokko L. Kinnula . 2008. Redox Imbalance and Lung Fibrosis. *Antioxidants & Redox Signaling* **10**:2, 249-252. [Citation] [Full Text PDF] [Full Text PDF with Links]