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PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPAR-γ) REGULATION OF INDUCTION OF INFLAMMATORY MEDIATORS BY ORGANIC DUST IN THP-1 MACROPHAGE CELLS

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PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPAR-γ) REGULATION OF INDUCTION OF INFLAMMATORY MEDIATORS BY ORGANIC DUST IN THP-1 MACROPHAGE CELLS

by

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LIST OF ABBREVIATIONS

15d-PGJ2: 15-Deoxy-Delta-12,14-prostaglandin

AFO: Animal feeding operation

AHR: Airway hyperresponsiveness

AhR: Aryl hydrocarbon receptor

AMPK: 5'-AMP-activated protein kinase

ANOVA: Analysis of variance

AP-1: Activator protein-1

BCP: Bromochloropropane

BSA: Bovine serum albumin

CAFO: Concentrated animal feeding operation

DCFDA: Dichlorodihydrofluorescein diacetate

DE: Organic dust extract

DMSO: Dimethyl sulfoxide

COPD: Chronic obstructive pulmonary disease

CCL: C-C motif chemokine ligand DHA: Docosahexaenoic acid

ECL: Enhanced chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

ERK: Extracellular signal-regulated kinase

EVs: Extracellular vesicles

HCl: Hydrochloric acid

HDAC: Histone deacetylases

HUVECs: Human umbilical vein endothelial cells

ICAM-1: Intercellular adhesion molecule 1

IL: Interleukin

JNK: c-Jun N-terminal Kinase

KC: Keratinocyte chemoattractant/ Keratinocyte-derived chemokine

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NaCl: Sodium chloride

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHBE: Normal human bronchial epithelial cells

NOD2: Nucleotide-binding oligomerization domain-containing protein-2

PAR: Protease-activated receptors

PBS: Phosphate-buffered saline

PMA: Phorbol 12-myristate 13-acetate

PPAR: Peroxisome proliferator-activated receptors

PPRE: PPAR response element

PTGS: Prostaglandin-endoperoxide synthase

PCR: Polymerase chain reaction

PKC: Protein kinase C

PVDF: Polyvinylidene fluoride

qRT-PCR: Quantitative real-time polymerase chain reaction

ROS: Reactive oxygen species

RXR: Retinoid X receptors

SDS-PAGE: Sodium dodecyl-sulfate polyacrylamide

SRA/ CD204: Scavenger receptor A/Cluster of differentiation 204

STAT-3: Signal transducer/ activator of transcription 3

TBST: Tris-buffered saline containing 0.1% Tween 20

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-α

ABSTRACT

Inhalation of organic dust is associated with the development of respiratory diseases such as bronchitis, hypersensitivity pneumonitis, asthma, and chronic obstructive pulmonary disease (COPD). Chronic inflammation due to increased cytokine production has been linked to the development and progression of respiratory diseases. Peroxisome proliferator-activated receptor gamma (PPAR-γ), a transcription factor belonging to the nuclear hormone receptor family, is known to modulate inflammatory responses. In this study, we investigated the involvement of PPAR-γ in the regulation of organic dust induction of inflammatory mediators in THP-1 macrophages. THP-1 monocytic cells were differentiated into macrophages using phorbol myristate acetate. The effects of PPAR-γ agonists and antagonists on the induction of IL-6 and TNF-a expression by poultry organic dust extract were investigated by ELISA and real-time qRT-PCR. The effects of PPAR-g agonist 15d-PGJ2 on ROS production and activation of MAPKs and NF-kB and STAT-3 activation were investigated by DCFDA labeling and western blotting respectively. We found that PPAR-γ agonist 15d-PGJ2 potently inhibited dust extract induction of TNF-α and IL-6 proteins by reducing mRNA expression. 15d-PGJ2 inhibited ROS levels and NF-κB and STAT-3 activation while concomitantly activating MAPKs. These data indicated that the inhibitory effects of 15d-PGJ2 on TNF-α and IL-6 may be mediated via reduced ROS and NF-κB and STAT-3 activation, suggesting that PPAR-γ could be targeted to attenuate organic dust-induced lung inflammation.

INTRODUCTION

CAFOs and Organic Dust

Livestock farming has undergone a notable transformation since the mid-twentieth century in the United States into large-scale mass production where livestock farms practice high-density animal farming operations (AFOs) known as concentrated animal feeding operations (CAFOs) to increase the efficiency of meat, milk, and egg production [1]. AFOs are defined by the U.S. Environmental Protection Agency (EPA) as agricultural enterprises where animals are kept and raised in a confined facilities [2]. CAFO is a large-scale industrial agricultural facility in which more than 1,000 animal units (an animal unit is the equivalent of 1,000 pounds of live animal weight) are confined for over 45 days in a year [2]. The poultry production industry, a significant component of the agricultural economy, is rapidly growing worldwide and employs several hundreds of thousands of workers in the United States alone [3]. Although CAFOs tend to be efficient and cost-effective, the environment in CAFOs tends to be contaminated with high levels of aerosolized organic dust [4]. As a result, agricultural workers at these operations can get exposed to the indoor environment for prolonged periods increasing their risk of developing respiratory symptoms and respiratory diseases.

Organic dust refers collectively to airborne and settled particulate matter of biological origin such as dust found in animal and agricultural farms [5]. Organic dust is a complex material containing animal dander, feathers, animal feed, microorganisms such as bacteria, viruses, and fungi, and their byproducts including endotoxin, peptidoglycan, and mycotoxin [4-7]. It also contains toxic gases such as methane, hydrogen sulfide, and ammonia that are produced by decomposing waste. Organic dust tends to be heterogenous in size ranging from 0.5 – 100 μm [8]. Although particles of various sizes may be deposited in the nose and pharyngeal region, only

particles with an aerodynamic diameter below 15 μm can enter the tracheobronchial tree and only particles with an aerodynamic diameter less than 7 μm can reach the alveoli [9]. Particles slightly larger than 20 μm diameter are referred to as inhalable particles, whereas particles with an aerodynamic diameter of less than 5 μm are referred to as respirable dust. Approximately 18% of total dust from poultry farm buildings constitutes respirable dust [6].

Studies have found high concentrations of microorganisms in the CAFO environment [4, 8]. Although microorganisms represent less than one percent of the number of aerosolized particles, they often may have significant negative health effects on CAFOs workers [8]. A study by Kiekhaefer et al. to characterize microbes in the environment of various types of CAFOs across seasons found moderately elevated culturable mold with fungal types *Cladosporium* and *Alternaria* predominant in the summer and fall and yeasts *Penicillium* and *Fusarium* mainly in the winter and spring [10]. Gram-positive bacteria are predominantly found in CAFOs; however, gram-negative bacteria also may have significant effects on the health of CAFO workers [6, 10]. Broiler poultry CAFO dust has been reported to contain the highest total bacterial concentrations than other CAFO dust [11].

Endotoxin, also known as lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and is the most frequently reported environmental airborne microbial contaminant in CAFOs [12]. In poultry farms, bacteria that are found in fecal matter, urine, litter, grain, and other vegetable matter in poultry feed have been found to be the source of endotoxin [6, 13]. LPS typically consists of a core oligosaccharide, an O-specific chain made up of repeating sequences of polysaccharides, and a lipid A component which is responsible for LPS's toxic effects [14]. Endotoxin level in CAFOs has been found not to be sufficient to account for lung inflammation in human subjects [15]. The highest concentrations of endotoxin were found

in poultry farms than in swine and cattle farms [11]. Protease activities found in aqueous extracts of swine [16] and poultry [17] farms dust have been found to be involved in the induction of inflammatory mediators suggesting that they may be one of the major components of animal farm dust promoting inflammatory responses. A recent study has demonstrated that poultry organic dust contains bacteria-derived extracellular vesicles (EVs) and that they induce inflammatory responses in lung epithelial cells in vitro and mouse lungs [18].

Organic Dust Exposure and Respiratory Symptoms and Diseases

Exposure to airborne dust in CAFOs has been found to be strongly associated with the development of both acute and chronic respiratory symptoms and diseases [19]. Agricultural workers, particularly poultry workers, experience a higher incidence of lower and upper respiratory tract symptoms and respiratory diseases such as asthma-like syndrome, bronchitis, asthma exacerbation, and organic dust toxic syndrome [20]. A high prevalence of chronic obstructive pulmonary disease (COPD) has also been found among agricultural workers [21].

Persistent or chronic inflammation has been linked to the development and progression of respiratory diseases [22]. Inflammatory responses protect the host against exogenous and endogenous danger signals resulting from systemic infection or biologically induced tissue damage which is characterized by the release of inflammatory mediators [23]. The resolution of inflammation is critical to the restoration of tissue homeostasis [24]. In the lung, acute and chronic inflammation can severely compromise vital gas exchange, and therefore, there are several control mechanisms in place to regulate the severity and duration of lung inflammation [25]. Failure to resolve inflammation and/or persistent exposure to the offending agent(s) can result in chronic inflammatory diseases [26].

Regulation of Induction of Inflammatory Mediators by Organic Dust

Respiratory symptoms and associated lung inflammatory responses have been investigated in human subjects exposed to CAFO dust. Acute exposure of naïve healthy human volunteers to swine [27, 28] and poultry [29] CAFO environments was found to induce airway hyperresponsiveness, fever, chills, and malaise. Exposure was found to markedly increase nasal and bronchoalveolar lavage (BAL) fluid levels of interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, IL-1α, and IL-1β, as well as granulocyte counts [27, 28]. Markers of inflammation were more pronounced in naïve subjects than CAFO workers indicating adaptation to inflammatory agents present in the CAFO environment [30].

Studies in mice to determine the effects of daily exposure to a range of doses of swine barn dust suspensions for 14 days found elevated airway hyperresponsiveness (AHR) and significantly higher levels of IL-1β, keratinocyte-derived chemokine (KC), and TNF-a in BAL fluid of mice exposed to the highest concentration of dust than controls [31]. Mice exposed to the highest concentrations of dust were also found to have higher counts of lymphocytes and monocytes but not neutrophils in the BAL fluid [31]. In another study, single and repetitive exposure of mice to swine facility dust extract showed increases in neutrophil and macrophage counts in BAL fluid [32]. BAL fluid TNF-α, IL-6, KC, and macrophage inflammatory protein-2 were also significantly increased after single and repetitive dust exposures but were dampened in 2 weeks dust extract-exposed mice than in mice with single exposure [32].

In vitro studies of treatments of A549 alveolar and Beas2B bronchial epithelial and THP-1 monocytic cells with poultry dust extract caused significant induction of several immune and inflammatory response genes including IL-8, IL-6, IL-1β, intercellular adhesion molecule 1 (ICAM-1), C-C motif chemokine ligand (CCL) 2, CCL5, toll-like receptor (TLR) 4, and

Prostaglandin-endoperoxide synthase (PTGS) 2 [33, 34]. Similar findings have also been reported for dust extracts of other agricultural dust. Exposure of normal human bronchial epithelial cells (NHBE), A549, and human alveolar macrophages to swine dust significantly increased IL-8 production [35]. Human bronchial epithelial cell exposure to hog dust extract [36] and feedlot dust extract [37] was noted to induce IL-8 and IL-6 release via a protein kinase C (PKC)-dependent pathway. Treatment of human peripheral blood monocytes and THP-1 monocytic cells to swine farm dust extracts increased TNF-α, IL-6, IL-8, and IL-10 levels [38]. Increases in cytokine levels were attenuated in cells that had been stimulated earlier. Increases in cytokines were associated with PKC activation, particularly PKCa and PKCe. Based on studies with human subjects, experimental animals, and lung cells in vitro, it is evident that organic dust exposure induces inflammatory responses and is associated with the development of respiratory symptoms and diseases. However, the mechanisms underlying the pathogenesis of respiratory diseases are not well understood.

Pattern recognition receptors, which recognize specific microbial components including endotoxin and peptidoglycans, such as TLR2 [39, 40], TLR4 [41], nucleotide-binding oligomerization domain-containing protein-2 (NOD2) [42], and scavenger receptor A (SRA)/cluster of differentiation 204 (CD204) [43], have been found to be involved in the control of the production of chemokines/cytokines and lung inflammatory responses in mice exposed to farm dust. Protease-activated receptors (PAR)-1 and -2 have been found to mediate the induction of inflammatory mediators by protease activities in organic dust [17, 44].

In A549 alveolar and Beas2B bronchial epithelial and THP-1 monocytic cells, induction of IL-8 by poultry organic dust extract was found to be dependent on the activation of PKC and mitogen-activated protein kinase (MAPK) [34]. Transcriptional mechanisms but not stabilization

of mRNA controls the induction of IL-8 gene expression. The increase of IL-8 promoter activity was dependent on the binding of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) transcription factors with NF-κB playing a major role in the induction [34]. Recent studies showed that signal transducer and activator of transcription 3 (STAT-3) [45] and aryl hydrocarbon receptor (AhR) [46] serve as positive transcriptional factors for the induction of inflammatory mediators by poultry organic dust extract.

Peroxisome Proliferator-Activated Receptors Gamma (PPAR-γ)

PPARs belonging to the nuclear hormone receptor superfamily are transcription factors that regulate a wide range of physiological functions such as energy and lipid metabolism, cancer, and inflammatory responses [47]. To date, three PPAR subtypes, namely PPAR-α, PPAR-β/δ, and PPAR-γ have been discovered [48]. In humans, PPAR-γ is encoded by the PPAR-γ gene on chromosome 3p25.2 [49]. The human PPAR-γ gene has 9 exons with four PPAR-γ splice variants (PPAR-γ 1-4) encodes for two protein isoforms PPAR-γ1 and PPAR-γ2 through alternative promoter usage [49]. The PPAR-γ1 isoform is expressed in nearly all cells, while PPAR-γ2 is limited to adipose tissue [50]. In the lung, PPAR-γ is expressed in smooth muscle and epithelial cells, and alveolar macrophages [51, 52].

PPARs regulate gene expression by forming a heterodimer with retinoid X receptors (RXRs) and binding to specific PPAR response elements (PPRE) [53], which contain repeats of the sequence AGGTCA, separated by one or two nucleotides in the promoter regions of target genes when activated by an appropriate ligand [54]. In the absence of the ligand, the PPAR/RXR complex is hindered from binding to PPRE as it is bound to transcriptional corepressors and histone deacetylases (HDACs) [55]. Upon activation, PPAR-γ undergoes conformational changes that facilitate recruitment of co-activators such as p300/CBP and p160 resulting in the

displacement of co-repressors and binding to target gene PPREs to induce transcription [55]. A schematic diagram depicting PPAR-γ activation is shown in Figure 1.

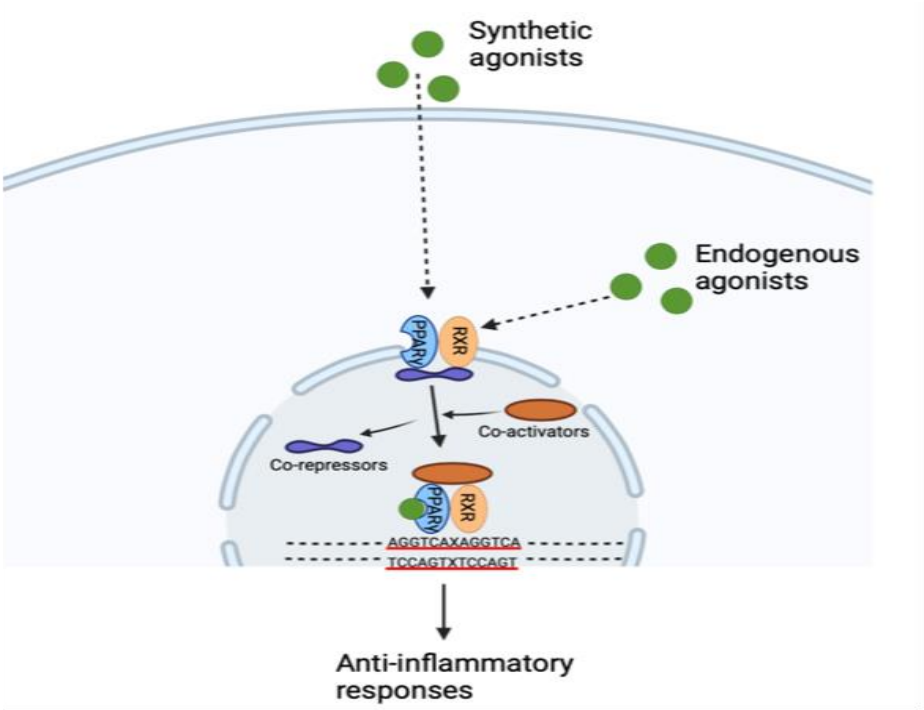

Figure 1. A schematic of the mechanism of ligand-induced PPAR-γ activation. The PPAR-RXR heterodimer is complexed to transcriptional co-repressors which prevent its binding to PPRE. Upon PPAR-γ activation by an agonist, conformational changes ensue, leading to the recruitment of co-activators such as p300/CBP and p160 resulting in the displacement of co-repressors and binding of PPAR-RXR to target gene PPRE and induction of transcription. Figure adapted from Wei *et al* [55].

PPAR-γ can be activated by a wide range of endogenous ligands and synthetic agonists. PPAR-γ is activated by fatty acid metabolites, such as 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), 9-hydroxyoctadecadienoic acid, nitrated fatty acids, and lysophosphatidic acids [47]. Other endogenous ligands such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) metabolites can also activate PPAR-γ [56]. The thiazolidinedione class of anti-diabetic drugs,

which include pioglitazone and rosiglitazone, are the most extensively studied and characterized PPAR-γ synthetic agonist [55].

PPAR-γ modulates inflammation in different organs including the lung, thereby serving as a potential target for the treatment of lung diseases [57]. PPAR-γ activation decreases the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 and inhibits the expression of adhesion molecules such as ICAM-1 [58]. The binding of PPAR-γ ligands negatively regulates inflammatory mediator gene expression by inhibiting transcription factors, such as AP-1, NF-κB, and STAT-3 [47]. Cytokine mixture induction of IL-6 and IL-8 expression in A549 and Beas2B lung epithelial cells was significantly inhibited by pre-treatment with PPAR-γ agonist rosiglitazone [59].

RESEARCH RATIONALE AND HYPOTHESIS

Rationale: Published studies have shown that PPAR-γ agonists exert anti-inflammatory effects, underlying their potential to treat inflammatory respiratory diseases. As there is no information on the involvement of PPAR-γ in the regulation of inflammatory responses induced by organic dust, studies are proposed to investigate the effects of PPAR-γ agonists on organic dust extract-induced inflammatory cytokines in THP-1 macrophages. As lung epithelial cells and lung (alveolar) macrophages contribute to the modulation of lung inflammatory responses, studies are proposed using THP-1 macrophages as a model macrophage cells.

Hypothesis: We hypothesized that PPAR-γ activation downregulates the induction of inflammatory mediators by poultry organic dust extract.

Experimental Objectives

The following experiments are proposed to address our hypothesis,

Experimental Objective 1. Determine the effects of PPAR-γ agonists and antagonists on the induction of inflammatory mediators by poultry dust extract (dust extract) in THP-1 macrophages.

Experimental Objective 2. Determine the effects of the PPAR-γ agonist identified in objective 1 on the activation of ERK, p38, and JNK MAPKs, NF-κB, and STAT-3 by dust extract.

MATERIALS AND METHODS

Organic Dust Extract Preparation

Broiler poultry dust settled on vertical surfaces was collected from a poultry farm in East Texas when the chickens were ~ 8 weeks of age. The farm is an indoor poultry facility equipped with tunnel ventilation in which chickens are floor-raised. The settled dust was collected into sterile plastic tubes and stored at -80°C until extraction. Organic dust extract was prepared as previously described by Gottipati et al [34]. The dust was incubated (1g/10 mL) in serum-free F12K medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) in an ultrasound water bath at room temperature for 10 min with periodic agitation. The mixture was centrifuged at 800 x g for 5 min at 4°C to first clear the suspension followed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was filtered using a 0.2 μm syringe filter and the filtrate was stored at -20°C. The concentration of this extract was arbitrarily assigned as 100%. The protein concentration of dust extract was determined by the Bradford method using bovine serum albumin (BSA) as the protein standard.

Cell Culture and Treatments

THP-1 monocytic cells (ATCC TIB-202) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.05 mM β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. THP-1 monocytic cells (~10^6 cells per mL) were plated in either 6-well or 12-well cell culture plates and differentiated into macrophages by treatment with 200 nM phorbol 12-myristate 13-acetate (PMA) for 2 days, washed, and rested in growth medium without PMA for 5 days according to the published procedure [60]. THP-1 macrophages were serum starved for 3 h in a serum-free RPMI 1640 medium containing antibiotics and antimycotics before the treatments in the same medium. THP-1 macrophages were

first treated with PPAR-γ agonist/ or antagonist for 1 h before treatment with dust extract. Control cells and cells treated with only dust extract were incubated with an identical concentration of the vehicle [dimethyl sulfoxide (DMSO)] used for dissolving PPAR-γ agonists (15-Deoxy-Delta-12,14-prostaglandin J2 (15d-PGJ2), GW1929, and Rosiglitazone) and PPAR-γ antagonist (GW9662).

ELISA

After treatment, cell medium was collected and centrifuged at 3000 rpm for 5 min at 4 °C and the supernatant was collected and stored at -80°C. The levels of IL-6 and TNF-α in the cell culture medium were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions.

MTS Cell Viability Assay

THP-1 monocytic cells (~ 10^5 cells/well) were plated in 96-well culture plates and differentiated into macrophages as described above. THP-1 macrophages were rinsed two times with serum-free RPMI 1640 medium containing antibiotics and antimycotics and incubated in the same medium for 3 h before treatment with PPAR-γ agonist or antagonist and dust extract. Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) according to the manufacturer's protocol.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

After treatments, THP-1 macrophage cells were washed twice with cold phosphate-buffered saline (PBS) and 40 µL ice-cold lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 x protease and phosphatase inhibitor cocktail, 1% Triton X-100 and 15% Glycerol) was added to each well of cell culture dishes. Cell lysates were collected by scraping cells into 1.5 mL tubes and incubated on ice for 10 min. The lysates were centrifuged at 13000 rpm (~ 15000 x g) for 10 min at 4°C. Supernatants were collected and their protein concentration was determined by Bradford assay. Equal amounts of cell lysate proteins (20-30 µg) were separated by SDS-PAGE on 10% NuPAGE Bis-Tris gels (Life Technologies) alongside protein molecular weight markers using MOPS as the running buffer. Separated proteins were transferred to Hybond-PVDF membranes (0.45 µm) (Millipore). Membranes were incubated in 5% non-fat dry milk in 1x tris-buffered saline containing 0.1% Tween 20 (TBST) with shaking at room temperature for 1 h. Membranes were washed in 1x TBST and incubated with primary antibody overnight at 4°C with shaking. Membranes were washed with 1x TBST and incubated with secondary antibody (anti-mouse IgG, HRP-linked antibody (7076, Cell Signaling Technology, 1:5000 dilution) or anti-rabbit IgG, HRP-linked antibody (7074, Cell Signaling Technology, 1:5000 dilution) diluted in 1x TBST for 1 h at room temperature with shaking. Membranes were washed with 1x TBST. Proteins were detected by the enhanced chemiluminescence (ECL) method (GE HealthCare Life Sciences) using Bio-Rad Chemidoc MP Imager and quantified using ImageLab software (Bio-Rad). Membranes were re-probed with actin antibodies to assess for equal loading and transfer of proteins.

RNA Isolation and Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using TRI-Reagent (Molecular Research Center). Cells were lysed with TRI reagent (1 mL/ well, 6-well dish) and samples were either stored at -80°C or immediately used for RNA isolation. For each lysate sample, 100 µL bromochloropropane (BCP) was added and mixed vigorously by hand. The mixture was incubated at room temperature for 10 min and centrifuged at 13,000 rpm for 15 min at 4°C; the top layer was carefully transferred to a new 1.5 mL tube and an equal amount of isopropanol and 2 µL of glycogen (5 µg/µL) were added. The samples were incubated at room temperature for 10 min and then centrifuged at 13,000 rpm for 8 min at 4°C. The supernatant was removed, and the RNA pellets were washed two times with 1 mL 75% ice-cold ethanol and centrifuged at 7,500 rpm for 5 min at 4°C. After washing, the pellets were allowed to air-dry and were dissolved in 45 µL DNase/ RNase-free water. RNA was incubated with TURBO DNase (Ambion) for 30 min at 37°C to remove genomic DNA, mixed with DNase inactivation reagent, and centrifuged at 10,000 rpm for 2 min. The supernatant containing RNA was precipitated by adding 0.3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. The mixture was incubated at -70°C for 30 min. RNA was pelleted by centrifugation at 13000 rpm for 15 min and washed twice with 1 ml 70% ethanol. RNA pellets were air-dried and dissolved in 20 µl nuclease-free water. RNA concentration was quantified by measuring absorbance at 260 nm using a nanodrop spectrophotometer (ThermoScientific) and its purity was checked by analyzing A260/A280 and A260/A230 ratios. Equal amounts of RNAs (1µg) were reverse transcribed to synthesize cDNA using the iScript Reverse Transcription kit (Bio-Rad) by incubation in a thermal cycler with the following conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The levels of cytokine and actin mRNAs were determined by real-time quantitative polymerase chain reaction (PCR) with TaqMan probe assays (Bio-Rad/Life Technology) using

CFX 96 Real-Time PCR Detection System (Bio-Rad) with the following reaction conditions – 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 30 seconds with 40 cycles. mRNA levels were normalized to actin mRNA levels and normalized gene expression data relative to the control sample arbitrarily set to 1 was obtained using CFX Manager Software (Bio-Rad).

Visualization of Intracellular ROS Generation in THP-1 Macrophage Cells

THP-1 macrophage cells were washed twice with serum-free RPMI medium without phenol red. Cells were first incubated with PPAR-γ agonist 10 µM 15d-PGJ2 for 1 h followed by 0.1% dust extract for 30 min. Afterward, cells were incubated with 10 µM dichlorodihydrofluorescein diacetate (DCFDA) for 30 min. Cells were washed twice with serum-free RPMI medium without phenol red and images were captured with ZOE Fluorescent Cell Imager (Bio-Rad). The fluorescent intensities of individual cells were measured using ImageJ software (NIH).

Statistics

Each experiment was performed at least three times independently and the statistical significance of the data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 9. All data are expressed as mean ± SE and a p-value of <0.05 was considered statistically significant.

RESULTS

Effects of PPAR-γ Agonists on Dust Extract Induction of TNF-α and IL-6 Protein Levels

Exposure of lung cells to poultry dust extract and its components has been found to induce inflammatory mediators [17, 18, 45, 61]. Activation of PPAR-γ has been found to modulate inflammatory responses in various organs and thus PPAR-γ may be a potential target for the treatment of inflammatory lung diseases [57]. To determine if PPAR-γ agonists modulate dust extract induction of inflammatory mediators, we first determined the effects of three well-known PPAR-γ agonists: 15d-PGJ2, GW1929, and Rosiglitazone on dust extract induction of TNF-α and IL-6. THP-1 macrophages were treated with different concentrations of PPAR-γ agonist followed by dust extract and the TNF-α and IL-6 protein levels were determined. Results showed that 15d-PGJ2 and GW1929 significantly reduced dust extract induction of IL-6 (Figure 1B). 15d-PGJ2 was also found to significantly reduce TNF-α whereas GW1929 did not. Rosiglitazone was found to dose-dependently inhibit dust extract-induced TNF-α and IL-6 levels, but the decreases were not statistically significant (Figure 2). From the results obtained, 15d-PGJ2 was found to be the most potent inhibitor of the induction of inflammatory mediators. Based on these results,15d-PGJ2 was selected for the rest of the studies.

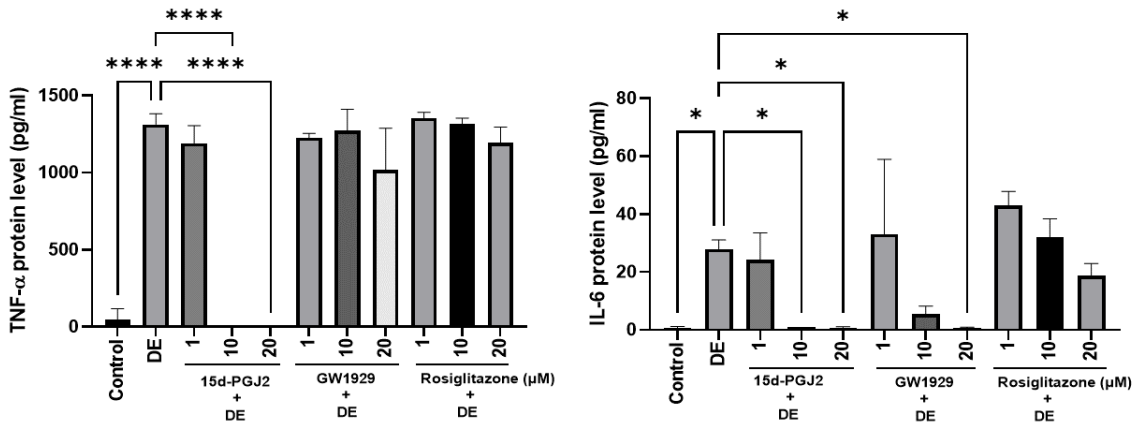

Figure 2. Effects of PPAR-γ agonists on the induction of inflammatory cytokines by organic dust extract in THP-1 macrophages. Cells were untreated (Control) or treated with a medium containing 1-20 μM 15d-PGJ2, GW1929, and Rosiglitazone for 1 h and incubated with 0.1% dust extract (DE) for 3 h. Levels of IL-6 and TNF-α in the cell medium were determined by ELISA. Data shown are mean ± SE (n = 3). *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

15d-PGJ2 Inhibits Dust Extract Induction of TNF-α and IL-6 Protein and mRNA Levels

15d-PGJ2 is an endogenous agonist of PPAR-γ [62]. Treatment of THP-1 macrophage cells with 10 μM of 15d-PGJ2 was found to significantly attenuate dust extract-induced IL-6 and TNF-α protein levels as shown in Figure 3. To determine whether the decreases in inflammatory mediator protein levels are due to alterations in gene expression, we investigated the effects of 10 μM 15d-PGJ2 on the mRNA levels of IL-6 and TNF-α in dust-extract treated cells. Results indicated that treatment with 10 μM 15d-PGJ2 significantly inhibited IL-6 and TNF-α mRNA levels (Figure 4). These data indicated that the attenuation of IL-6 and TNF-α levels induced by dust extract is due to the inhibition of gene expression. Adverse effects of treatments on cell viability could confound the interpretation of the results obtained. Therefore, the effects of treatments on THP-1 macrophage cell viability were assessed by MTS assay. It was found that treatments had no effect on cell viability (Figure 5).

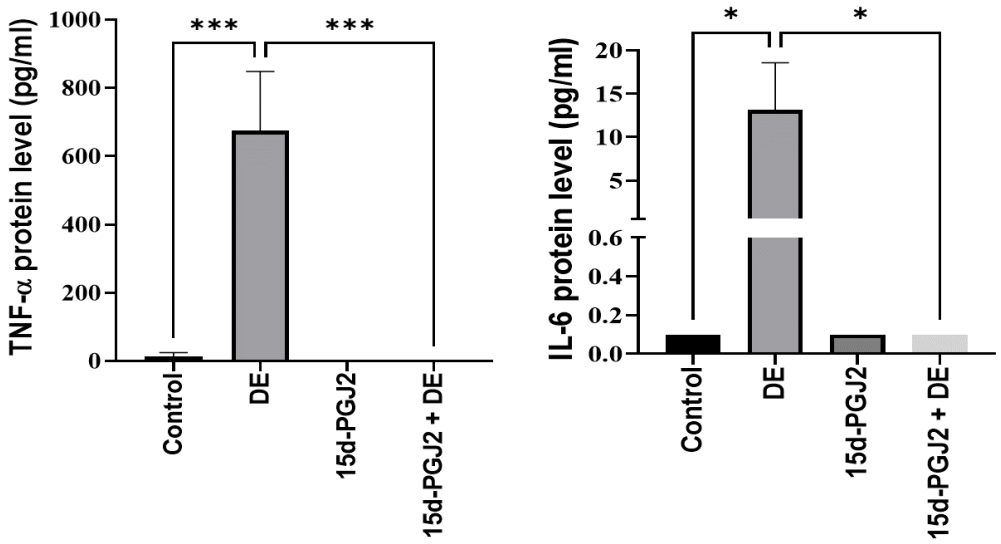

Figure 3. PPAR-γ agonist 15d-PGJ2 inhibits dust extract induction of TNF-α and IL-6 protein levels. Cells were untreated (Control) or treated with a medium containing 10 µM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 3 h. Levels of TNF-α and IL-6 in the cell medium were determined by ELISA. Data shown are mean ± SE (n = 3 – 6). *p < 0.05, and ***p < 0.001.

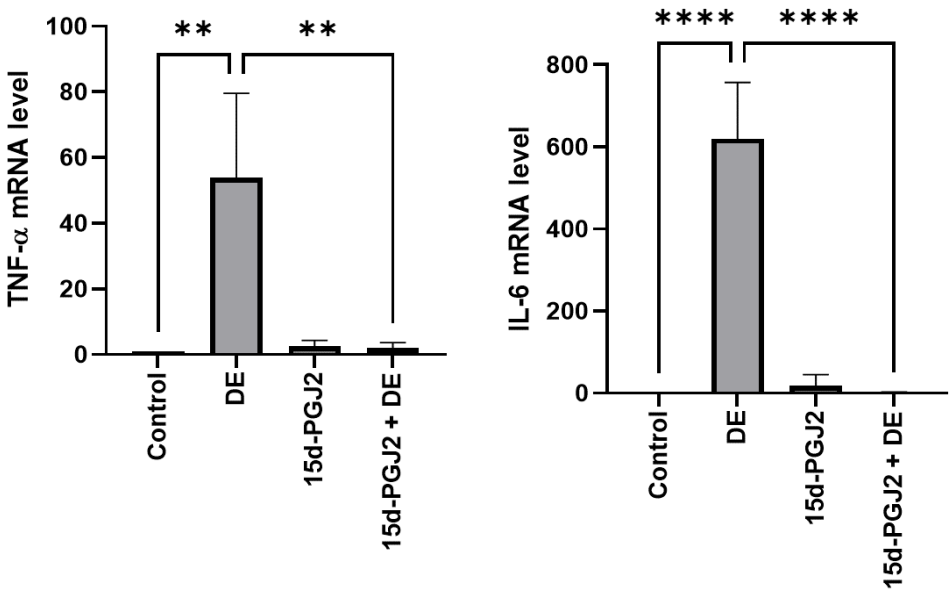

Figure 4. PPAR-γ agonist 15d-PGJ2 inhibits dust extract induction of TNF-α and IL-6 mRNA levels. Cells were untreated (Control) or treated with a medium containing 10 μM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 3 h. IL-6, TNF-α, and actin mRNA levels were determined by qRT-PCR and IL-6, and TNF-α levels were normalized to actin levels. Data shown are mean ± SE (n = 3). *p < 0.05 and ****P < 0.0001.

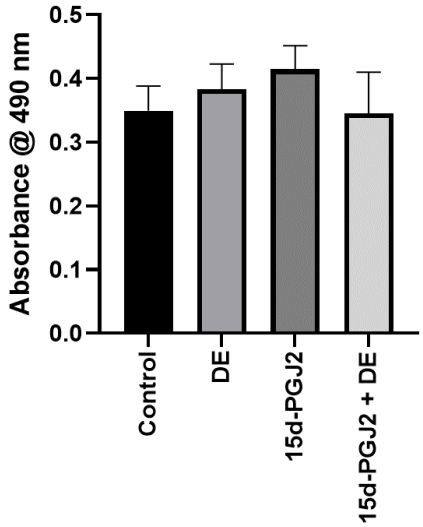

Figure 5. PPAR-γ agonist 15d-PGJ2 does not affect THP-1 macrophage cell viability. Cells were untreated (Control) or treated with a medium containing 10 μM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 3 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Data shown are means ± SE (n=4).

PPAR-γ Antagonist GW9662 has no Effect on the Induction of IL-6 and TNF-α Protein Levels

PPAR-γ antagonist GW9662 has no effect on the induction of IL-6 and TNF-α protein levels. GW9662 acts as a potent, irreversible, and selective PPAR-γ antagonist with an IC_{50} value of 3.3 nM [63]. We determined the dose-response effects of GW9662 on dust extract induction of IL-6 and TNF-α protein levels. Results indicated that GW9662 did not have any significant effects on the induction of IL-6 and TNF-α protein levels by the dust extract (Figure 6).

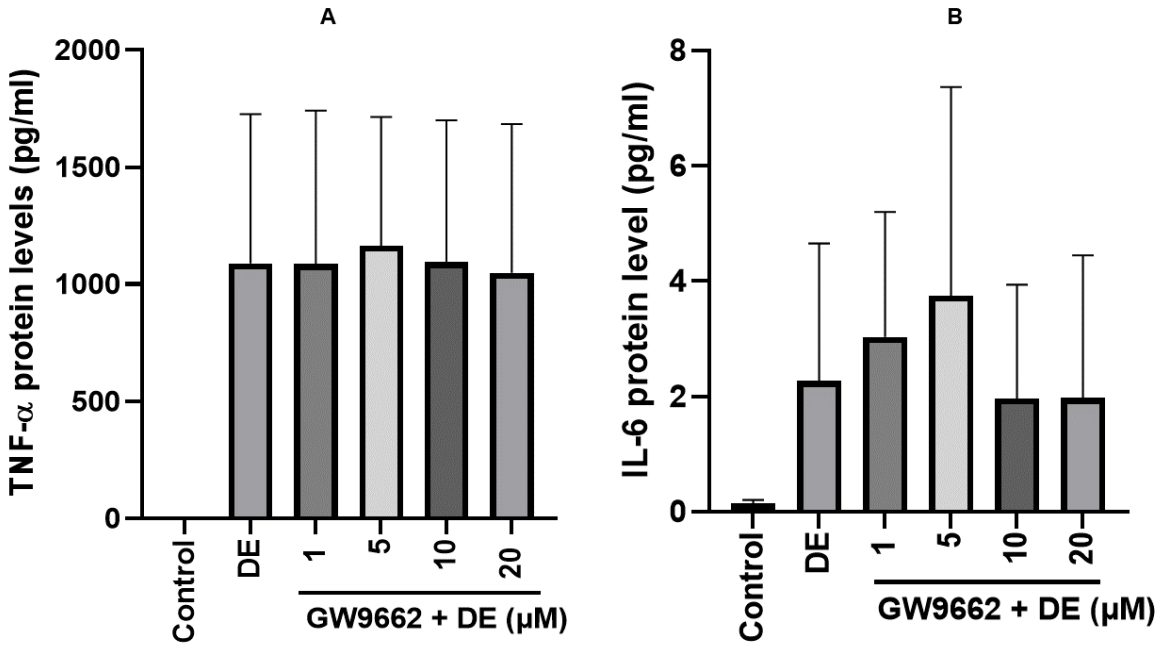

Figure 6. PPAR-γ antagonist GW9662 has no effect on the induction of IL-6 and TNF-α protein levels. Cells were untreated (Control) or treated with a medium containing 1-20 μM GW9662 for 1 h and incubated with 0.1% dust extract (DE) for 3 h. Levels of TNF-α and IL-6 in the cell medium were determined by ELISA. Data shown are mean ± SE (A: n = 3, B: n = 2 - 3).

Effects of 15d-PGJ2 and GW9662 on the Induction of IL-6 and TNF-α Protein Levels

To further investigate whether the activation of the PPAR-γ receptor is involved in the inhibitory effect of 15d-PGJ2 on dust extract induction of IL-6 and TNF-α levels, we co-treated THP-1 macrophage cells with both 15d-PGJ2 and GW9662. It was noted that the 15d-PGJ2-mediated decrease of IL-6 and IL-8 production was not reversed by the PPAR-γ antagonist, GW9662 (Figure 7). Similar results were obtained with a different PPAR-γ antagonist T0070907 (data not shown).

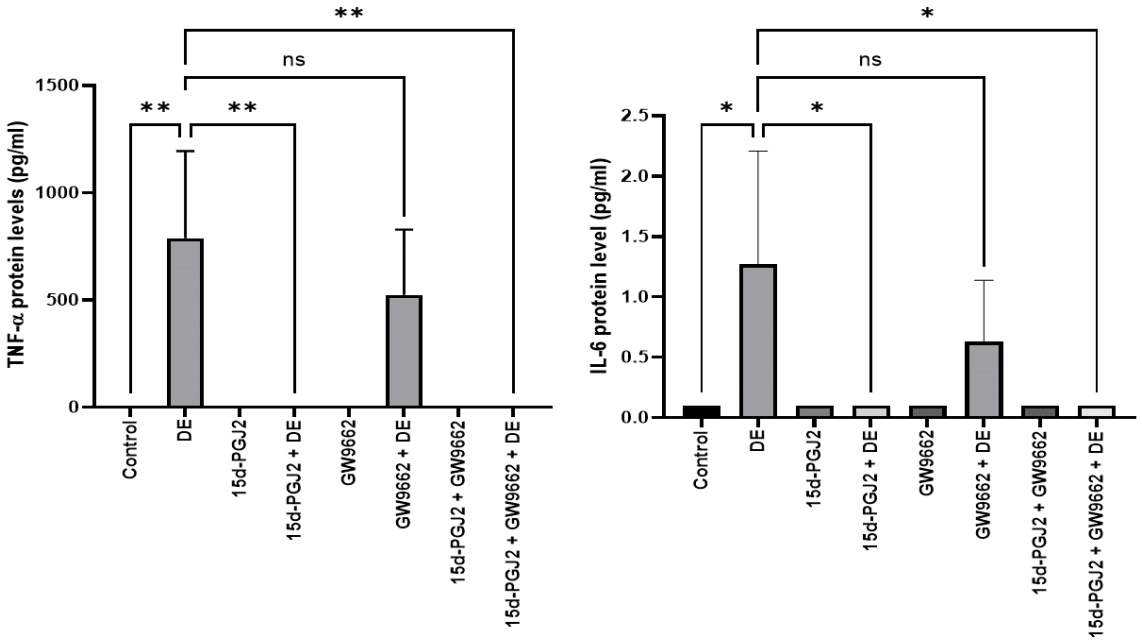

Figure 7. Effects of PPAR-γ agonist 15d-PGJ2 and antagonist GW9662 on the induction of IL-6 and TNF-α protein levels. Cells were untreated (Control) or treated with a medium containing 10 μM 15d-PGJ2 and 10 μM GW9662 for 1 h and incubated with 0.1% dust extract (DE) for 3 h. The levels of TNF-α and IL-6 protein in the cell medium were determined by ELISA. Data shown are mean ± SE (n = 3). *$p < 0.05$ and **$P < 0.01$.

15d-PGJ2 Inhibits Dust Extract Induction of Reactive Oxygen Species (ROS)

Studies from our lab have demonstrated that poultry dust extract controls the induction of immune and inflammatory mediator expression via increased production of ROS [17, 45]. To understand the mechanisms by which 15d-PGJ2 inhibits the induction of TNF-α and IL-6, we investigated the effect of 15d-PGJ2 on dust extract induction of ROS. The results showed that 15d-PGJ2 significantly inhibited ROS induction (Figure 8).

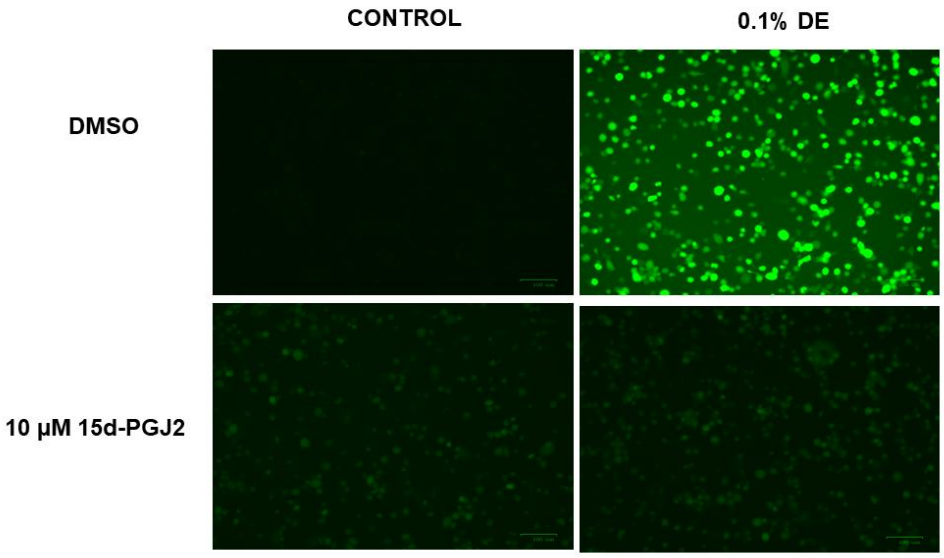

Figure 8. PPAR-γ agonist 15d-PGJ2 inhibits dust extract induction of ROS. Cells were untreated (Control) or treated with a medium containing 10 μM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 30 min. ROS generation was visualized by DCFDA labeling. Representative images of 3 independent experiments are shown.

15d-PGJ2 inhibits dust extract activation of NF-κB and STAT-3

NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway, largely based on the role of NF-κB in the expression of proinflammatory genes [64]. Activation of STAT proteins plays a critical role in the control of innate immune and inflammatory responses [65]. Among the various STAT proteins, STAT-3 activation has been implicated in the development of acute and chronic lung injury [66]. Our laboratory previously found that NF-κB [17] and STAT-3 [45] activation regulates the induction of inflammatory mediators by dust extract in airway epithelial cells. Therefore, the effect of 15d-PGJ2 on NF-κB and STAT-3 activation was investigated. Results indicated that NF-κB and STAT-3 activation by dust extract was significantly attenuated by 15d-PGJ2 as shown in Figures 9 and 10.

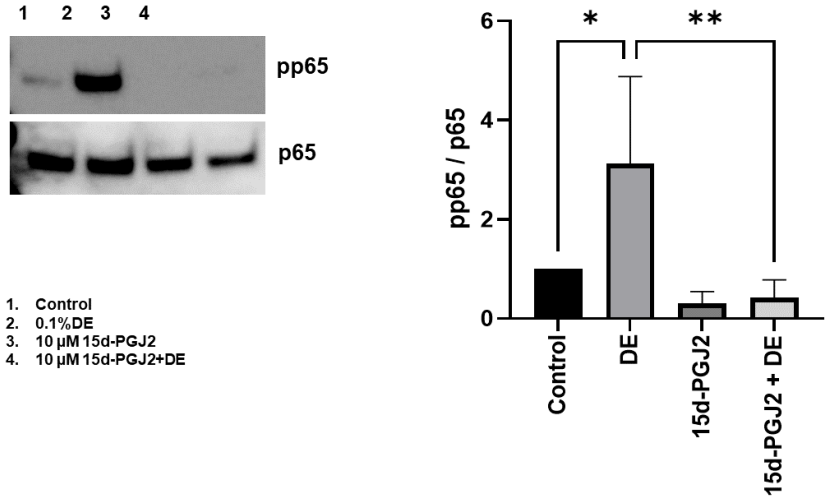

Figure 9. PPAR-γ agonist 15d-PGJ2 inhibits dust extract activation of NF-κB. Cells were untreated (Control) or treated with a medium containing 10 µM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 30 min. The levels of phosphorylated and total NF-κB p65 were determined by western blotting. Data shown are mean ± SE (n = 4). *$p < 0.05$, and **$p < 0.001$.

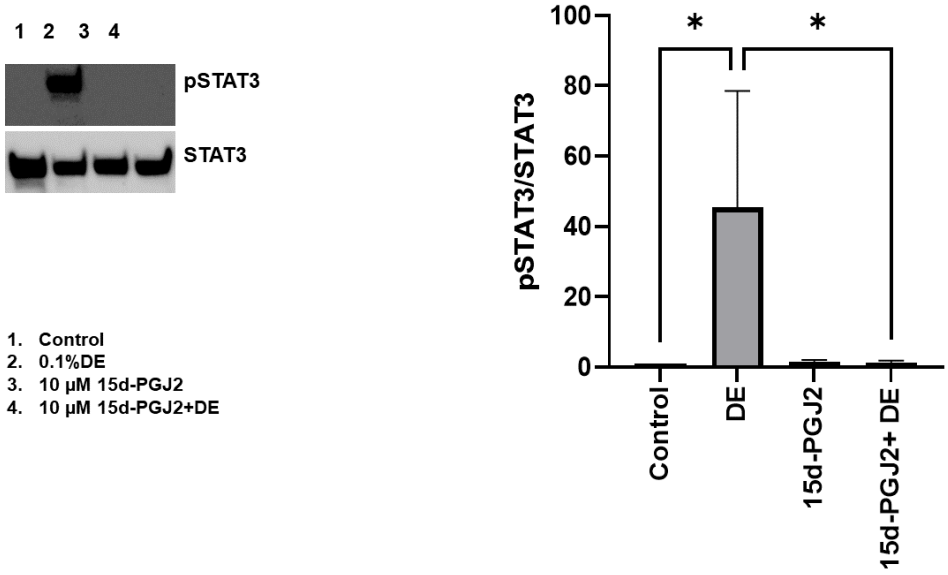

Figure 10. PPAR-γ agonist 15d-PGJ2 inhibits dust extract activation of STAT-3. Cells were untreated (Control) or treated with a medium containing 10 µM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 2 h. The levels of phosphorylated and total STAT-3 were determined by western blotting. Data shown are mean ± SE (n = 3). *$p < 0.05$.

Effects of 15d-PGJ2 on Dust-Induced Activation of Mitogen-Activated Protein Kinases (MAPKs)

Studies from our lab have demonstrated MAPKs (p38, JNK, and ERK) signaling pathways mediate the induction of inflammatory mediators by poultry dust extract in A549 and Beas2B lung epithelial and THP-1 monocytic cells [34]. We, therefore, investigated the effects of 15d-PGJ2 on the activation of p38, JNK, and ERK MAPKs in THP-1 macrophages. It was found that 15d-PGJ2 did not inhibit but rather induced the activation of p38, JNK, and ERK MAPKs (Figure 11).

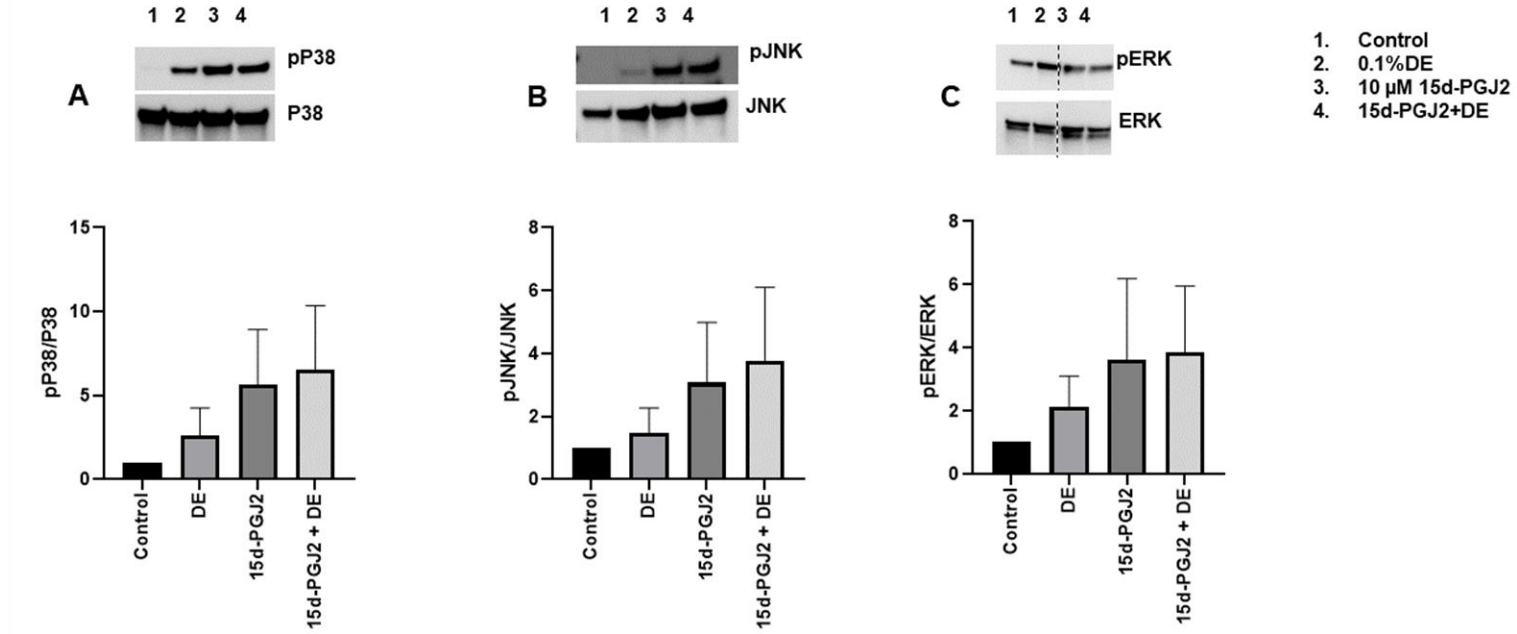

Figure 11. Effects of PPAR-γ agonist 15d-PGJ2 on dust extract induced activation of MAPKs. Cells were untreated (Control) or treated with a medium containing 10 µM 15d-PGJ2 for 1 h and incubated with 0.1% dust extract (DE) for 30 min for (A and B) and 10 min for (C). Levels of phosphorylated and total p38 (A), JNK (B), and ERK (C) were determined by western blotting. Data shown are mean ± SE (n = 3).

DISCUSSION

Exposure to airborne dust is strongly associated with the development of both acute and chronic respiratory symptoms and diseases [19]. Chronic exposures to organic dust are linked to the development of bronchitis, asthma, hypersensitivity pneumonitis, organic dust toxic syndrome, and COPD [5, 20]. A major characteristic of these diseases is persistent or chronic lung inflammation [22]. Previous studies have indicated that in vitro exposure of lung epithelial cells, monocytic cells, and macrophages to poultry organic dust and its components is associated with increased production of inflammatory mediators such as TNF-α, IL-6, and IL-8 [17, 18, 34]. The induction of inflammatory mediators by poultry dust is mediated by a cellular pathway involving oxidative stress (increase in ROS production) [17, 18]. Studies to determine the effects of poultry dust extract on the induction of IL-8 expression in A549 and Beas2B lung epithelial and THP-1 monocytic cells indicated that activations of PKC and MAPK signaling pathways mediate induction [34]. Transcriptional factors NF-κB, AP-1, and STAT-3 are important for the induction of inflammatory mediators by poultry dust extract [18, 34, 45].

Activation of PPAR-γ is known to negatively modulate inflammation in many organs including the lung [57]. Studies of the effects of various synthetic and endogenous PPAR-γ ligands (activators) have been performed to understand the role of PPAR-γ in lung inflammatory diseases. For example, PPAR-γ activator rosiglitazone was found to attenuate IL-6 and IL-8 levels in a cell culture model of acute lung injury induced by LPS [59]. The anti-inflammatory effects of PPAR-γ agonists have also been demonstrated in asthma [67] and COPD [68].

The role of PPAR-γ in the modulation of organic dust-induced lung inflammation has not been studied previously. In this study, we investigated whether PPAR-γ regulates the induction of

inflammatory mediators by organic dust in THP-1 macrophages using its agonists and antagonists. As alveolar macrophages contribute to the modulation of lung inflammatory responses, we employed THP-1 macrophages as a cell model to study the role of PPAR-γ in the modulation of inflammatory cytokines induced by organic dust extract. Although THP-1 macrophages may not fully recapitulate alveolar macrophage cell responses, they may still provide valuable information that can serve as the basis for future studies. We tested our hypothesis by determining the effects of a structurally diverse array of PPAR-γ agonists, 15d-PGJ2, GW1929, and rosiglitazone, on dust extract-induced TNF-α and IL-6 (Figure 1). We found that 15d-PGJ2 significantly inhibited the induction of TNF-α and IL-6 more than GW1929 and rosiglitazone. Whereas GW1929 inhibited IL-6 levels at 10 and 20 μM, rosiglitazone only had modest inhibitory effects. Both GW1929 and rosiglitazone did not appear to inhibit TNF-α levels. The reasons for the observed differences in the inhibitory effects of these PPAR-γ agonists are not known, and further investigation is needed. Whether the observed effects are the result of cell-specific actions of the PPAR-γ agonist needs further investigation. Our findings of the potent inhibitory effects of 15d-PGJ2 are consistent with previously published studies. In a study aimed at exploring the anti-inflammatory potential of PPAR-γ agonists, 15d-PGJ2, thiazolidinedione (TZD), and non-TZD, on LPS-induced IL-6 and TNF-α in human peripheral blood monocytes and macrophages, it was found that only 15d-PGJ2 exerted inhibitory effects [69]. Hinz *et al* reported that 15d-PGJ2 but not ciglitazone attenuated LPS-induced inflammatory mediators' gene expression in human peripheral blood monocytes [70].

PPAR-γ antagonist, GW9662 did not have any significant effect on dust extract induced TNF-α and IL-6 (Figure 6) suggesting that 15dPGJ2 activation of PPAR-γ may not be necessary for the inhibition of induction of TNF-α and IL-6 levels. Additionally, GW9662 did not reverse

the inhibitory effects of 15d-PGJ2 on the induction of TNF-α and IL-6 levels. These findings raise the question of whether the observed inhibitory effects are dependent on PPAR-γ or occur independently. PPAR-γ antagonists have been reported to partially reverse as well as being ineffective against the inhibitory effects of 15d-PGJ2. Whereas GW9662 was found to partially reverse the inhibitory effects of 15d-PGJ2 on macrophage activation by LPS [71], it failed to reverse the anti-inflammatory effects of 15d-PGJ2 in rat primary astrocytes [72]. Hinz et al found that the inhibitory actions of 15d-PGJ2 on the expression of several pro-inflammatory genes in LPS-stimulated human blood monocytes were not reversed by PPAR-γ antagonist, BADGE [70]. A study by Chawla et al also noted that the attenuation of LPS-induced proinflammatory cytokines in wildtype macrophages by 15d-PGJ2 and various TZDs was equally effective in PPAR-γ-deficient macrophages [73], suggesting that the inhibitory effects of 15d-PGJ2 and various TZDs on inflammatory cytokine production may be PPAR-γ independent. In human umbilical vein endothelial cells (HUVECs), rosiglitazone was demonstrated to reduce glucose-induced oxidative stress via inhibition of NADPH oxidase that was mediated by AMPK activation, but independently of PPAR-γ [74].

To elucidate the cellular pathways by which 15d-PGJ2 inhibits inflammatory mediators, we investigated the effects of 15d-PGJ2 on dust extract induction of ROS and activation of NF-κB and STAT-3 transcription factors. We found that 15d-PGJ2 significantly attenuated dust extract-induced ROS production (Figure 7). As increased production of ROS is important for the induction of inflammatory mediators [17, 45], 15d-PGJ2 inhibition of dust extract-induced ROS suggests a possible mechanism to understand the suppressive effects of 15d-PGJ2 on the induction of cytokines. In mouse models of allergic airway disease induced by ovalbumin, PPAR-γ agonists rosiglitazone and pioglitazone attenuated ROS production and nuclear NF-kB levels [67].

We found that 15d-PGJ2 significantly suppressed dust extract activation of NF-κB and STAT-3 indicating that the suppression of TNF-α and IL-6 levels may be due to inhibition of gene transcription. PPAR-γ agonists are known to inhibit inflammatory mediators' gene expression by regulating transcription factors such as NF-κB, and STAT-3. A study to elucidate the molecular mechanism of action by which PPAR-γ agonists suppress inflammation showed that 15d-PGJ2 and rosiglitazone inhibited the activation of STAT-1 and STAT-3 as well as JAK-1 and JAK-2 in activated astrocytes and microglia independently of PPAR-γ [75]. Straus et al reported that 15d-PGJ2 direct inhibition of NF-κB signaling may contribute to its negative regulation of inflammation [76]. 15d-PGJ2 was shown to inhibit IkB kinase and NF-kB p65 via covalent modification of critical cysteine residues [76].

We found that PPAR-γ agonist, 15d-PGJ2 did not inhibit dust extract activation of p38, JNK, and ERK MAPKs but, rather induced their phosphorylation indicating that MAPKs may not mediate the inhibitory effects of 15d-PGJ2 on dust extract induction of IL-6 and TNF-α. A study to investigate molecular mechanisms mediating the anti-tumor effects of 15d-PGJ2 in different osteosarcoma cell lines reported that 15d-PGJ2 induced the activation of p38, JNK, and ERK MAPKs via increased production of ROS [77]. Activation of H-Ras via covalent modification by 15d-PGJ2 was reported to mediate MAPK activation [78].

In summary, our studies have shown that the PPAR-γ agonist, 15d-PGJ2 potently attenuated organic dust induction of IL-6 and TNF-α levels by inhibiting their mRNA expression. Inhibition by 15d-PGJ2 was associated with the inhibition of ROS production and inhibition of NF-κB and STAT-3 activation. Additional studies are necessary to understand if the anti-inflammatory effects of 15d-PGJ2 are mediated by PPAR-γ or by other mechanisms.

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VITA

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