Taylor & Francis
Taylor & Francis Group

Journal of Toxicology and Environmental Health, Part A

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

PGJ2 Inhibition of LPS-induced Inflammatory Mediator Expression from Rat Alveolar Macrophages

Dale W. Porter , Michael Wolfarth , Shih-Houng Young , Murali K. Rao , Terence Meighan , Mark Barger , Michael E. Andrew & Linda J. Huffman

To cite this article: Dale W. Porter , Michael Wolfarth , Shih-Houng Young , Murali K. Rao , Terence Meighan , Mark Barger , Michael E. Andrew & Linda J. Huffman (2007) PGJ2 Inhibition of LPS-induced Inflammatory Mediator Expression from Rat Alveolar Macrophages, Journal of Toxicology and Environmental Health, Part A, 70:23, 1967-1976, DOI: 10.1080/15287390701549260

To link to this article: https://doi.org/10.1080/15287390701549260

Journal of Toxicology and Environmental Health, Part A, 70: 1967–1976, 2007

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390701549260

Taylor & Francis
Taylor & Francis Group

PGJ2 Inhibition of LPS-induced Inflammatory Mediator Expression from Rat Alveolar Macrophages

Dale W. Porter
National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, West Virginia, USA
Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia, USA

Michael Wolfarth, Shih-Houng Young, Murali K. Rao, Terence Meighan, Mark Barger, and Michael E. Andrew
National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, West Virginia, USA

Linda J. Huffman
National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, West Virginia, USA
Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia, USA

Studies suggested that 15-deoxy-Δ-^{12,14}-prostaglandin J_2 (PGJ2) may exert anti-inflammatory effects, including in the lung. Thus, in vitro studies were conducted to (1) investigate whether PGJ2 inhibited the production of inflammatory mediators from lipopolysaccharide (LPS)-exposed primary rat alveolar macrophages (AM), and (2) investigate possible mechanisms underlying PGJ2-mediated inhibition of inflammatory mediator production. These studies determined that PGJ2 inhibited LPS-induced nitric oxide (NO) production in a concentration- and time-dependent manner. PGJ2-mediated inhibition of NO, as well as of tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-2 (MIP-2), was also determined to be dependent on the time of addition of PGJ2 relative to LPS, and suggested the PGJ2 inhibitory mechanism is an early event. PGJ2 was shown not to interfere with binding or internalization of LPS by AM, indicating this was not responsible for PGJ2 inhibitory effects. Another possible mechanism underlying PGJ2-mediated inhibition was via peroxisome proliferator-activated receptor-γ (PPAR-γ). However, biochemical studies suggested that PGJ2-mediated inhibition was not occurring through PPAR-γ dependent mechanism, and molecular studies further established that both LPS and PGJ2 decrease PPAR-γ mRNA expression. A third possible mechanism underlying PGJ2-mediated inhibition was by alteration of nuclear factor (NF)-κB. Molecular studies confirmed that LPS stimulated NF-κB mRNA expression, and PGJ2 reduced this stimulation, which is consistent with PGJ2 effect on LPS-induced production of NO, TNF-α and MIP-2. Thus, data in this study established that PGJ2 inhibited LPS-induced inflammatory mediator production in rat AM, and this inhibition is mediated, at least in part, by reducing the expression of NF-κB mRNA.

It is well documented that prostaglandin production increases in response to diverse stimuli, and produces a wide spectrum of biological responses (Narumiya et al., 1999). Studies suggested that the prostaglandin 15-deoxy-Δ-^{12,14}-prostaglandin J_2 (PGJ2) may exert anti-inflammatory effects. In vitro studies with primary mice peritoneal macrophages determined that interferon-γ (IFN-γ)-induced expression of inducible nitric oxide synthase (iNOS) mRNA and nitrite production was inhibited in a dose-dependent manner by PGJ2 (Ricote et al., 1998). Similarly, interleukin-10 (IL-10) and interleukin-12 (IL-12) production by primary rat peritoneal macrophages after exposure to lipopolysaccharide (LPS) was inhibited in a dose-dependent manner by PGJ2 (Azuma et al., 2001). The hypothesis that PGJ2 may exert anti-inflammatory effects was also supported by in vivo studies that demonstrated an anti-inflammatory role

Received 12 March 2007; accepted 28 April 2007.
The authors express their appreciation to Delores Prugh for her technical assistance and to Dr. Vincent Castranova for his critical discussions during the conduct of the study.
The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.
Address correspondence to Dale W. Porter, PhD, National Institute for Occupational Safety and Health, 1095 Willowdale Road, M/S 2015, Morgantown, WV 26505, USA. E-mail: dporter@cdc.gov

for PGJ2 in various disease models, for example, inflammatory bowel disease (Su et al., 1999), Alzheimer's disease (Landreth & Heneka 2001), and arthritis (Kawahito et al., 2000).

In regard to pulmonary inflammation, specifically a carrageen-induced model of acute lung injury, PGJ2 accumulation in alveolar macrophages (AM) coincided with the peak of inflammation through resolution (Mochizuki et al., 2005). In addition, exogenously administered PGJ2 reduced pulmonary inflammation, as evidenced by a decrease in polymorphonuclear leukocytes (PMN), in mice that had endogenous PGJ2 synthesis inhibited by a COX-2 inhibitor (Mochizuki et al., 2005). These observations suggested that PGJ2 may also play an anti-inflammatory role in the lung, and this effect may be mediated by AM.

Several lines of investigation suggested that one possible mechanism underlying PGJ2 anti-inflammatory action is via activation of the peroxisome proliferator-activated receptor-γ (PPAR-γ). Synthetic PPAR-γ ligands were shown to produce a dose-dependent inhibition of IFN-γ induced expression of iNOS mRNA and nitrite production by primary mice peritoneal macrophages (Ricote et al., 1998), whereas a PPAR-γ agonist inhibited LPS-induced IL-10 and IL-12 production by primary rat peritoneal macrophages (Azuma et al., 2001). Further support for this mechanism was obtained using the RAW 264.7 mouse-monocyte cell line. RAW 264.7 cells have very low levels of PPAR-γ, and PGJ2 had little to no effect on IFN-γ induced expression of iNOS mRNA and nitrite production. However, PGJ2 inhibited IFN-γ stimulated iNOS expression in RAW 264.7 cells transfected with a PPAR-γ expression plasmid, indicating that PGJ2 inhibits induction of the iNOS promoter via a PPAR-γ dependent mechanism (Ricote et al., 1998).

Nuclear factor-κB (NF-κB) regulates the transcription of many inflammatory mediators that are involved in lung inflammatory responses (Park & Christman, 2006). PGJ2 was demonstrated to alter NF-κB activity by (1) inducing synthesis of IκB (Castrillo et al., 2000), or(2) binding to and inhibiting the action of IκB kinase (Rossi et al., 2000), or (3) inhibiting NF-κB binding to DNA (Straus et al., 2000). Thus, another possible mechanism underlying PGJ2 inhibition of LPS-induced inflammatory mediator production is that PGJ2 may alter NF-κB activity.

The purpose of the present study was to investigate whether PGJ2 inhibits the production of inflammatory mediators from LPS-exposed primary rat AM. In addition, these in vitro studies investigated possible mechanisms underlying PGJ2 inhibition.

MATERIALS AND METHODS

Animals

Animals used in these experiments were male Sprague-Dawley [Hla:(SD) CVF] rats weighing 200–225 g obtained from Hilltop Lab Animals (Scottdale, PA). The animals were housed in an AAALAC-accredited, specific-pathogen-free, environmentally controlled facility. Rats were monitored to be free of endogenous viral pathogens, parasites, mycoplasms, *Helicobacter*, and CAR *Bacillus*. Rats were housed one per cage in ventilated cages, which were provided HEPA-filtered air. Alpha-Dri virgin cellulose chips and hardwood Beta-chips were used as bedding. The rats were maintained on Harlan Teklad Global Rodent Diet 2018 (Indianapolis, IN) and tap water provided ad libitum. All animal procedures were approved by the Animal Care and Use Committee of the National Institute for Occupational Safety and Health.

Bronchoalveolar Lavage and Cell Differentials

To obtain naive AM, bronchoalveolar lavage (BAL) was conducted. Rats were euthanized with an ip injection of sodium pentobarbital (100 mg/kg body weight) and exsanguinated by cutting the abdominal vena cava. Next, a tracheal cannula was inserted and BAL was performed through the cannula using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) supplemented with 5.5 m*M* D-glucose (pH 7.4). A total of 80 ml of lavage fluid was collected. BAL cells were isolated by centrifugation (650×g, 5 min, 4°C). The acellular supernatants from the lavage samples were decanted and discarded. BAL cells isolated from the same rat were pooled after resuspension in HEPES-buffered medium (10 m*M* *N*-[2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 145 m*M* NaCl, 5.0 m*M* KCl, 1.0 m*M* $CaCl_2$, 5.5 m*M* D-glucose; pH 7.4), centrifuged a second time (650×g, 5 min, 4°C), and the supernatant was decanted and discarded. The BAL cell pellet was then resuspended in HEPES-buffered medium and placed on ice. BAL cell counts of AM and PMN were obtained using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL). Cytospin preparations of BAL cells were made using a cytocentrifuge (Shandon Elliot Cytocentrifuge, London). The cytospin preparations were stained with modified Wright–Giemsa stain and cell differentials were determined by light microscopy. The BAL cells obtained were predominantly AM, with PMNs representing <1% of the BAL cell population.

AM Cell Culture

AM were cultured at a density of 0.5×10^6 cells/ml in 12-well plates using Eagle's minimum essential medium (EMEM) media supplemented with 10 m*M* HEPES, 10% heat-inactivated fetal bovine serum, 1 m*M* glutamine, and 100 U/ml penicillin and streptomycin, at 37°C in 5% CO_2. Some cultures also contained lipopolysaccharide (LPS, *Escherichia coli* strain 0111:B4), PGJ2 (Cayman Chemicals, Ann Arbor, MI), ciglitazone (CG; BIOMOL International, Plymouth Meeting, PA), or GW9662 (Cayman Chemicals, Ann Arbor, MI). Dimethyl sulfoxide (DMSO) was used as solvent for PGJ2, ciglitazone, and GW9662, with final concentration of 0.1% (v/v) DMSO in cell culture media. After 18 h in culture, the samples were transferred to centrifuge tubes, centrifuged (650×g, 10 min, 4°C), and the cell-free supernatants (BAL cell-conditioned media) were removed. The AM-conditioned media were aliquoted into multiple samples and frozen at −80°C for later analyses.

BAL Fluid NOx Concentration

In this study, NOx was defined as the total of nitrite (NO_2^-) plus nitrate (NO_3^-) in a sample. NOx was determined using a method previously described by our lab (Porter et al., 2002). Briefly, the nitrate reduction reaction consisted of 50 m*M* HEPES, 5 m*M* flavine adenine dinucleotide, 0.1 m*M* reduced nicotinamide adenine dinucleotide phosphate, 0.2 U nitrate reductase (Roche Molecular Biochemicals, Indianapolis, IN), and BAL cell-conditioned media in a total volume of 1 ml. This reaction was incubated with gentle mixing at 37°C for 30 min. At the end of the incubation, the reaction solution was diluted with water and nitrite was determined by flow injection analysis colorimetry at 540 nm using the Griess reaction.

Cellular LDH Release

Cell culture plates were centrifuged (650×g, 10 min, 4°C) and the cell-free supernatants were transferred to 1.5-ml microcentrifuge tubes and placed on ice. The cell culture plates were washed by addition of ice-cold PBS, followed by centrifugation (650×g, 10 min, 4°C). The wash supernatant was decanted, and the cells were lysed by addition of 0.5 ml PBS containing 0.2% (v/v) Triton X-100. After 30 min of rocking at 4°C, the cell lysates were removed and placed in 1.5-ml microcentrifuge tubes on ice. Lactate dehydrogenase (LDH) activities were determined on cell-free supernatants and cell lysates by monitoring the LDH-catalyzed oxidation of lactate to pyruvate coupled with the reduction of NAD^+ at 340 nm using a commercial assay kit (Roche Diagnostics Systems, Montclair, NJ) and a COBAS MIRA Plus Auto analyzer (Roche Diagnostic Systems, Montclair, NJ).

Cytokine Determinations

Tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-2 (MIP-2) were determined in BAL cell-conditioned media using commercial enzyme-linked immunosorbent assay (ELISA) assay kits (BioSource, Camarillo, CA).

Western Blot Analysis of PPAR-γ

BAL cells were isolated from naive rats as already described. The BAL cell pellet was lysed with extraction buffer (50 m*M* Tris-HCL (pH 7.5), 2 m*M* EDTA, 100 m*M* NaCl, 1% (v/v) Igepal CA-630, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 m*M* phenylmethylsulfonyl fluoride). The BAL cell lysate was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Novex precast gels (Invitrogen, Carlsbad, CA). The proteins were transferred to a nitrocellulose membrane using semidry transfer blotter (Hoefer Scientific Instruments, San Francisco, CA). The blot was blocked with 5% nonfat dry milk in 20 m*M* Tris-HCl (pH 7.4) buffer containing 0.15M NaCl (TBS) for 1 h at room temperature. The blot was washed with TBS 0.1% Tween 20 (TBST) 3 times (5 min each) and incubated with primary polyclonal anti-PPAR-γ antibody (sc-7273, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The blot was washed 3 times with TBST and exposed to goat anti-mouse immunoglobulin (Ig) G–horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibody for 1 h at room temperature. After washing, the blot was exposed to streptavidin–horseradish peroxidase for 20 min. The blot was washed four times with TBST and the antigen–antibody complex was detected on a photographic film using electrochemical luminescence (ECL) reagent (Amersham, Arlington Heights, IL).

Quantitation of mRNAs by Real-Time RT/PCR

Quantitation of mRNA levels was measured using the ABI 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA). Total RNA was isolated using RNAqueous-4PCR kits (Ambion, Austin, TX) from AM (~0.5×10^6 cells). About 0.5 ug of the DNAse I-treated RNA was reverse transcribed (RT), using Superscript II (Life Technologies, Gaithersburg, MD). The cDNA generated was diluted 1:100, and 7.5 μl was used to conduct the polymerase chain reaction (PCR) using Roche Universal Probes in a total volume of 25 μl of FastStart TaqMan Probe Master Mix (Roche Applied Science, Indianaplois, IN). The comparative C_T (threshold cycle) method was used to calculate the relative concentrations (User Bulletin 2, ABI PRISMR 7700 Sequence Detector, PE Applied Biosystems, Foster City, CA). Briefly, the method involves obtaining the C_T values for the substance of interest, normalizing to a housekeeping gene (18 S ribosomal RNA in the present case), and deriving the fold increase compared to control unexposed cells. The primers and the probes used were: (1) NF-κB (p50 subunit) (ENSRNOT 00000036838), probe number 25, Sense: CCC ACT TGC TGC CTC TCT, Xsense: GTC ACA CAC GCT GTC ATT ATC TC, (2) PPAR-γ (ENSRNOT00000051858), probe number 7, Sense: CAG GAA AGA CAA CAG ACA AAT CA, Xsense: GGG GGT GAT ATG TTT GAA CTT G, and (3) 18 S ribosomal RNA (accession number X01117), probe number 55, Sense: GGT GCA TGG CCG TTC TTA, Xsense: TCG TTC GTT ATC GA ATT AAC C.

Statistical Analysis

Since the experiments in this study were randomized complete block designs, the analyses comparing means across the various treatment conditions were performed using mixed models with a random effect for blocks and fixed effects for treatments. Each block represents one of four animals from which cells were taken to perform the experiments. Mixed model analyses were performed using the SAS MIXED procedure. Linear dose-response analyses were also performed with mixed models including dose as a continuous fixed effect. In some instances there were treatment group combinations for which the response was equal to zero across all blocks, resulting in zero variance. In cases where this comparison was

not of specific interest these groups were excluded from analyses. In cases where there were cells with zero variance, and contrasts were of interest, nonparametric Kruskal–Wallis tests were performed, with Monte Carlo estimation of exact *p* values appropriate for tied data and small samples, using SAS PROC NPAR1WAY. Significance was set at $p < .05$.

RESULTS

PGJ2 Inhibits LPS-induced NOx

To determine whether PGJ2 inhibited LPS-induced NO production, AM-conditioned media NOx obtained from LPS-exposed AM were compared to AM preincubated with PGJ2 prior to LPS exposure (Figure 1). In the absence of PGJ2, 0.1, 50, and 100 μg/ml LPS induced a significant increase in NOx versus DMSO vehicle control. At all three concentrations LPS tested, preincubation with 10 μ*M* PGJ2 produced a significant inhibition of LPS-induced NO production, indicated by decreased NOx concentrations. These data indicate that PGJ2 inhibited LPS-induced NO production from rat primary AM.

Concentration Dependence of PGJ2 Inhibition of LPS-induced NOx

To determine the concentration-dependence of PGJ2 inhibition, NOx production by LPS-exposed AM (0.1 μg/ml LPS) with PGJ2 concentration ranging from 0 to 10 μ*M* was determined (Figure 2). In the absence of PGJ2, LPS exposure induced a significant increase in NOx. With increasing PGJ2 concentration, LPS-induced NOx progressively decreased, but remained significantly elevated versus control, except for the 10 μ*M* PGJ2 concentration, where there was no statistical difference between 0- and 0.1-μg/ml LPS exposure groups. Comparison of NOx levels from LPS-exposed AM with 0.1% DMSO (100.4 ± 12.2 nmol/0.5×10^6 AM) and 0 % DMSO (110.6 ± 11.5 nmol/0.5×10^6 AM) determined they were not statistically different, thus indicating 0.1% DMSO exerted no effect on AM response to LPS.

LDH release was determined in order to investigate whether concentration-response curve of PGJ2 inhibition was due to PGJ2-induced cytotoxicity (Table 1). These LDH determinations indicated that PGJ2-induced cytotoxicity was low and did

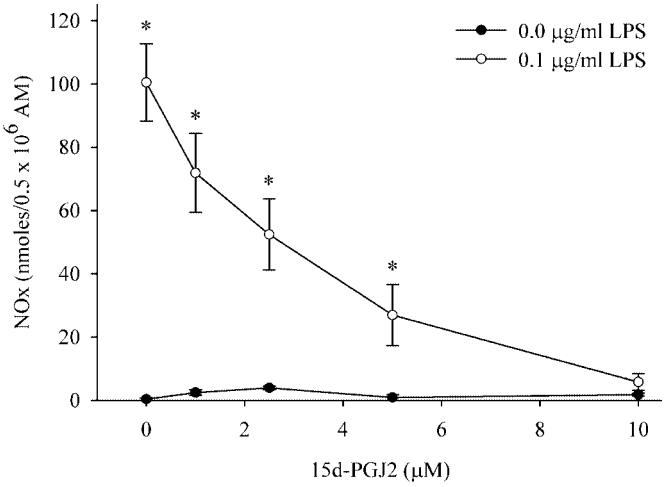

FIG. 2. PGJ2 inhibition of LPS-induced NOx is concentration dependent. AMs (0.5×10^6 cells/ml) were incubated with vehicle or PGJ2 (1–10 μ*M*) for 2 h prior to addition of 0.1 μg/ml LPS. Eighteen hours after LPS exposure, AM-conditioned medium was isolated. At each PGJ2 concentration, an asterisk indicates a significant ($p < .05$) difference between 0 and 0.1 μg/ml LPS doses. Values represent means ± SE ($n=4$).

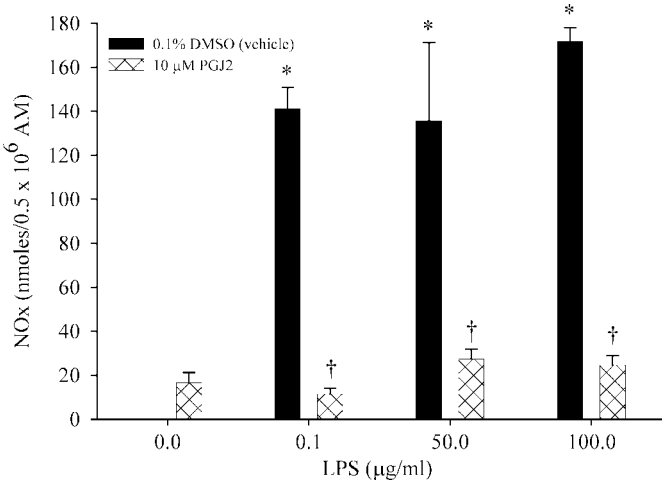

FIG. 1. PGJ2 inhibits LPS-induced NOx production by AM. AM (0.5×10^6 cells/ml) were incubated with vehicle or 10 μ*M* PGJ2 for 2 h prior to addition of LPS. Eighteen hours after LPS exposure, AM-conditioned medium was isolated. For LPS, an asterisk indicates a significant ($p < .05$) difference from vehicle control. At each concentration of LPS, a dagger (†) indicates a significant difference between 0 and 10 μM PGJ2 groups. Values represent means ± SE ($n=3$).

TABLE 1
PGJ2 Inhibition of LPS-Induced NOx Is Not Due to Cytotoxicity

	Supernatant LDH (% of total cellular LDH)	
PGJ2 (μ*M*)	0 μg/ml LPS[a,b]	0.1 μg/ml LPS[a,b]
0	1.64 ± 1.40	9.55 ± 1.29
1	7.13 ± 2.45	N.D.
2.5	0.37 ± 0.37	4.17 ± 2.51
5	N.D.	11.51 ± 2.84
10	4.61 ± 1.86	9.83 ± 1.48

[a]Values represent mean ± SE ($n=4$).
[b]N.D., not detected.

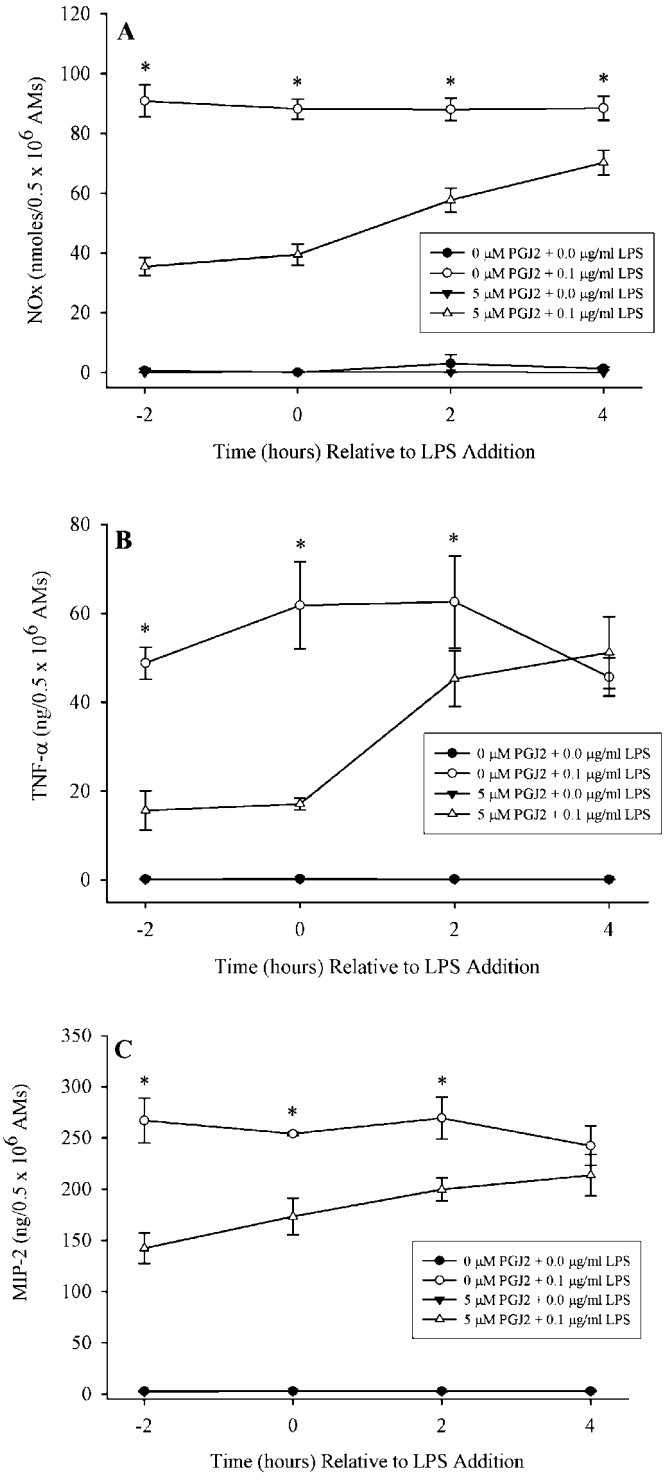

FIG. 3. PGJ2 inhibits LPS-induced NO, TNF-α and MIP-2 production. AM (0.5×10^6 cells/ml) were incubated with vehicle or 0.1 μg/ml LPS; 5 μ*M* PGJ2 was added to the cell culture either 2 h prior to (–2 h), simultaneously with (0 h), or 2 and 4 h after addition of LPS. Eighteen hours after LPS exposure, AM-conditioned medium was isolated. For each time, an asterisk indicates a significant ($p < .05$) difference between assays containing 0.1 μg/ml LPS alone and LPS and PGJ2. Values represent means ± SE ($n=4$).

not follow a linear concentration-response or the same concentration-response curve as PGJ2 inhibition of NO production. Thus, PGJ2-induced cytotoxicity was not responsible for the observed PGJ2-inhibiton of LPS-induced NO production.

Inhibition of LPS-Induced NOx, TNF-α and MIP-2 Is Affected by Time of Exposure to PGJ2

To investigate the effect of time of addition of PGJ2 relative to LPS on inflammatory mediator production, PGJ2 was added to AM cell cultures either 2 h before LPS exposure (–2 h), simultaneously with LPS exposure (0 h), or 2 and 4 h after LPS exposure. For LPS-induced NO production (Figure 3A), PGJ2 produced a significant inhibition in NOx, regardless of time of addition. However, PGJ2 inhibition was greatest when added 2 h before LPS, and least when added 4 h after LPS. For TNF-α (Figure 3B) and MIP-2 (Figure 3C), similar results were obtained. However, in contrast to NOx, when PGJ2 was added 4 h after LPS exposure, no statistical difference was detected between 0 and 5 μ*M* PGJ2 exposure groups, indicating PGJ2 was not inhibitory when added 4 h after LPS exposure for either TNF-α or MIP-2.

Effect of PGJ2 on LPS Binding to AMs

To investigate the possibility that PGJ2 inhibition of LPS-induced inflammatory mediators is due to a competition between PGJ2 and LPS for binding sites on AM, a flow cytometric experiment was conducted. The AM gate was drawn on a side scatter versus forward scatter plot from BAL cells obtained from a naive rat (Figure 4A). Addition of FITC-labeled LPS produced a shift of AM cells from an LPS-FITC negative region to an LPS-FITC positive region (Figure 4, B and C). Addition of PGJ2 did not affect either background AM binding or LPS binding to AM (Figure 4, D and E). The uptake of LPS-FITC was also not affected by the presence of PGJ2 (Figure 5).

PPAR-γ Is Present in Rat AMs

Western blot analyses determined that PPAR-γ is present in naive rat AM, with molecular mass of approximately 70 kD (Figure 6).

The Effect of Ciglitazone on LPS-Induced NOx

The effect of ciglitazone (CG), a PPAR-γ ligand, was investigated on LPS-induced NO production by AM (Figure 7). At all concentrations of CG tested, LPS-induced NO production remained significantly increased versus vehicle controls. Furthermore, NO production measured from LPS-exposed AM did not decrease significantly with increasing CG concentration. Thus, CG did not inhibit LPS-induced NO production.

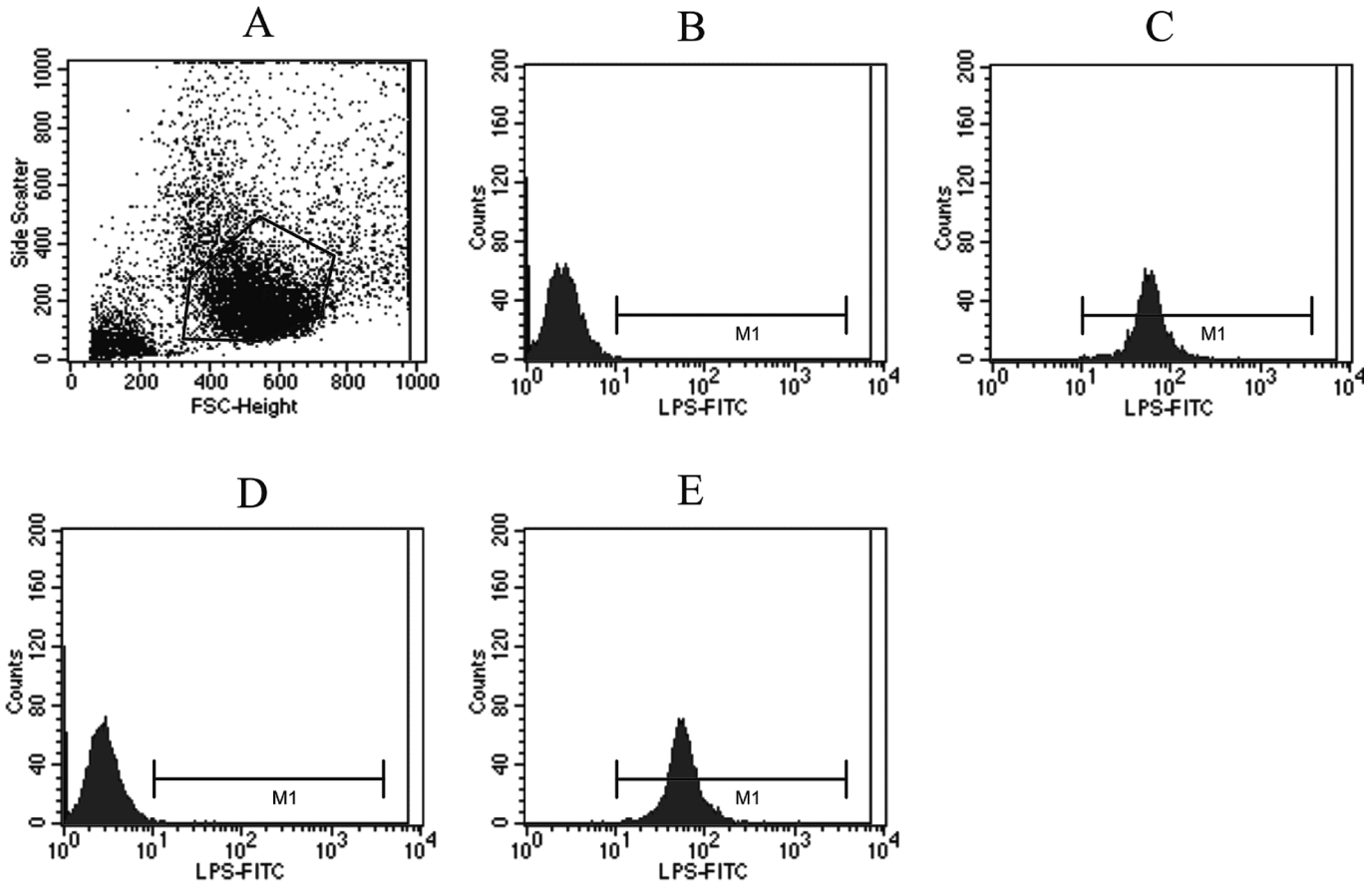

FIG. 4. Flow-cytometric analysis demonstrates PGJ2 does not affect LPS binding to AM. (A). SSC versus FSC dot plot of AM (gate was drawn based on AM cells population). (B). AM cells only in LPS-FITC histogram. Only 0.5% of AM cells were gated in FITC positive in M1 region, which demonstrates low background fluorescence intensity was observed in AM cells. (C). AM and LPS-FITC demonstrates that approximately 99% of AM were gated in FITC positive after incubation with LPS-FITC, indicating active uptake of LPS by AM. (D). AM+PGJ2 shows 0.5% of AM were gated in FITC positive in M1 region. (E) AM+PGJ2+LPS-FITC. Approximately 99% of AM were gated in FITC positive after incubation with PGJ2 and LPS-FITC, suggesting binding of LPS to AM was not affected by PGJ2.

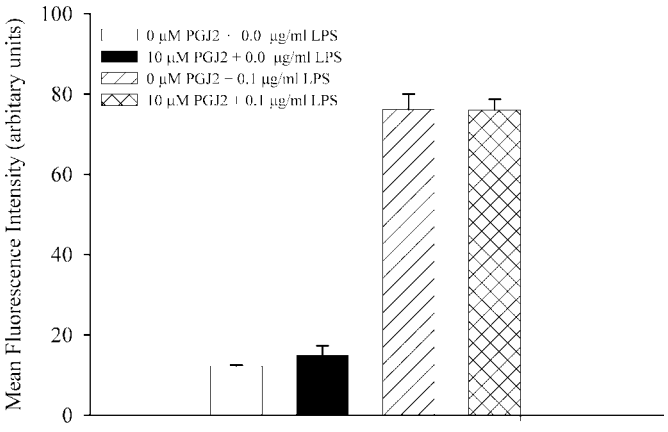

FIG. 5. The effect of adding PGJ2 does not affect LPS binding with AM. There were no differences in either control or LPS-FITC-treated groups with or without PGJ2. The data represent mean ± SE (n=4).

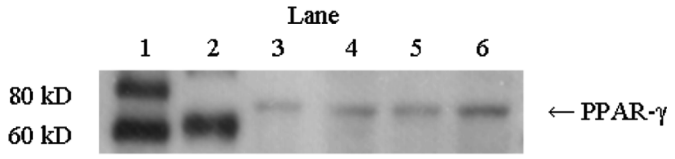

FIG. 6. Western blot analyses of PPAR-γ in AMs. AMs were obtained from naive rats by BAL and Western blot analyses were conducted to determine if PPAR-γ is present. Lane 1, molecular weight markers. Lane 2, 100 μg mouse heart extract (positive control). Lane 3, 50 μg BAL cell extract sample 1. Lane 4, 100 μg BAL cell extract sample 1. Lane 5, 50 μg BAL cell extract sample 2. Lane 6, 100 μg BAL cell extract sample 2.

GW9662 Effect on PGJ2 Inhibition of LPS-Induced NOx

The effect of GW9662, a PPAR-γ antagonist, was investigated on LPS-induced NO production by AM (Figure 8). AM exposed to 0 or 20 μ*M* GW9662 had very low levels of NOx,

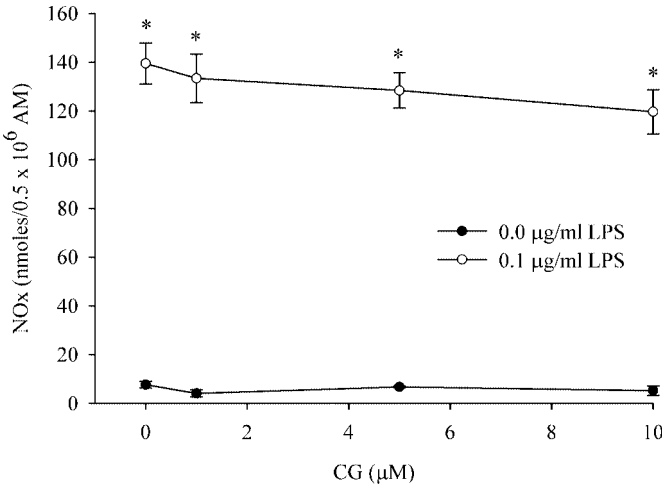

FIG. 7. Ciglitazone, a PPAR-γ ligand, does not have the same effect as PGJ2. AM (0.5×10^6 cells/ml) were incubated with vehicle or CG (1–10 μ*M*) for 2 h prior to addition of 0.1 μg/ml LPS. Eighteen hours after LPS exposure, AM-conditioned medium was isolated. For each CG concentration, an asterisk indicates a significant ($p < .05$) difference between 0 and 0.1 μg/ml LPS groups. Values represent means ± SE ($n=4$).

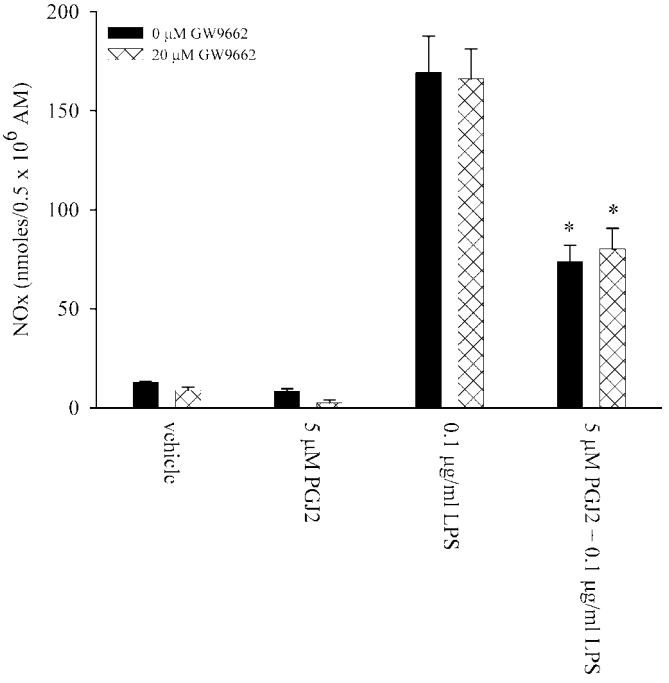

FIG. 8. GW9662, a PPAR-γ antagonist, does not block PGJ2 inhibition of LPS-induced NO production. AM (0.5×10^6 cells/ml) were incubated with vehicle, GW9662 (20 μ*M*), PGJ2 (5 μ*M*), and/or LPS (0.1 μg/ml). Relative to the time of addition of LPS, GW9662 was added 4 h before and PGJ2 was added 2 h before. Eighteen hours after LPS exposure, AM-conditioned medium was isolated. An asterisk indicates a significant ($p < .05$) difference between 0 and 0.1 μg/ml LPS groups for a given concentration of GW9662. Values represent means ± SE ($n=3$–4).

and comparison of these values indicated they were not significantly different. Similarly, AM exposed to 5 μ*M* PGJ2, with either 0 or 20 μ*M* GW9662, also had low NOx values, and comparison of these values indicated they were not significantly different. Thus, neither 20 μ*M* GW9662 nor 5 μ*M* PGJ2 alone, nor 20 μ*M* GW9662 and 5 μ*M* PGJ2 in combination, stimulated NOx production by AM. For AM cultures containing 0.1 μg/ml LPS, comparison of effects of 0 and 20 μ*M* GW9662 concentrations on NO production were not significantly different. For AM cultures containing 0.1 μg/ml LPS and 5 μ*M* PGJ2, comparison of effects of 0 μ*M* and 20 μ*M* GW9662 on NO production determined they were not significantly different. Thus, GW9662 itself had no effect on LPS-induced NOx production; nor did GW9662 affect PGJ2 inhibition of LPS-induced NOx production.

Effect of PGJ2 and LPS on mRNA Levels of PPAR-γ and NF-κB

The effect of PGJ2 and LPS on mRNA levels of PPAR-γ was investigated by RT-PCR analyses (Figure 9). PPAR-γ mRNA in AM exposed to 10 μ*M* PGJ2 alone (10 μ*M* PGJ2+0 μg/ml LPS) was significantly lower in comparison to levels in control AM (0 μ*M* PGJ2+0 μg/ml LPS), indicating that PGJ2 inhibited expression of PPAR-γ. LPS alone (0 μ*M* PGJ2+0.1 μg/ml LPS) also produced significant inhibition of PPAR-γ mRNA in comparison to control (0 μ*M* PGJ2 and 0 μg/ml LPS); the magnitude of LPS inhibition was larger than that produced by PGJ2. Comparison of PPAR-γ mRNA in AM exposed to LPS alone (0 μ*M* PGJ2+0.1 μg/ml LPS) to AMs incubated with LPS and PGJ2 (10 μ*M* PGJ2+0.1 μg/ml LPS) showed no difference in the magnitude of inhibition, thus indi-

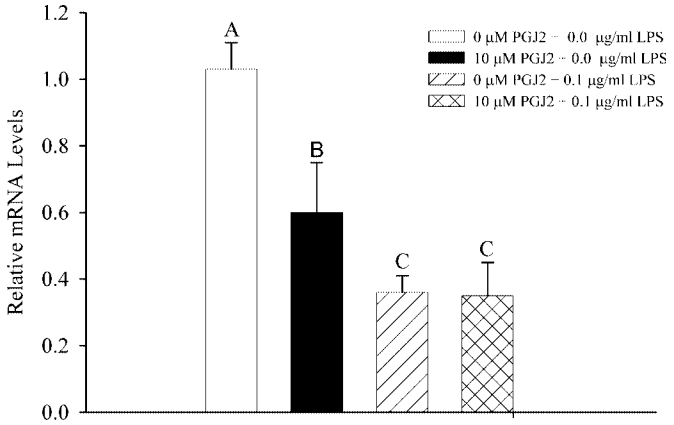

FIG. 9. PGJ2 and LPS affect mRNA expression of PPAR-γ. AM (0.5×10^6 cells/ml) were incubated with vehicle, PGJ2 (10 μ*M*) and/or LPS (0.1 μg/ml). PGJ2 was added 2 h before addition of LPS; 2 h after LPS exposure, AM-conditioned medium was aspirated and RNA isolated from AMs as described in Materials and Methods section. Bars with different letters are significantly different. Values represent means ± SE ($n=6$).

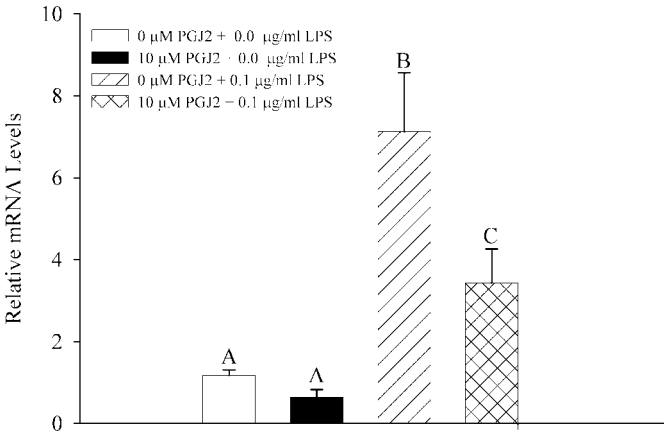

FIG. 10. PGJ2 and LPS affect mRNA expression of NF-κB p50 subunit. AM (0.5×10^6 cells/ml) were incubated with vehicle, PGJ2 (10 μ*M*), and/or LPS (0.1 μg/ml). PGJ2 was added 2 h before addition of LPS; 2 h after LPS exposure, AM-conditioned medium was aspirated and RNA isolated from AMs as described in Materials and Methods section. Bars with different letters are significantly different. Values represent means ± SE ($n=6$).

cating that PGJ2 did not prevent LPS-induced inhibition, nor were the LPS and PGJ2 inhibitory effects additive.

The effect of PGJ2 and LPS on mRNA levels for the p50 subunit of NF-κB was also investigated by RT-PCR analyses (Figure 10). NF-κB p50 subunit mRNA in AM exposed to 10 μ*M* PGJ2 alone (10 μ*M* PGJ2 + 0 μg/ml LPS) was not different in comparison to controls (0 μ*M* PGJ2 and 0.1 μg/ml LPS), indicating that PGJ2 did not affect basal expression of NF-κB p50 subunit. LPS alone (0 μ*M* PGJ2+0.1 μg/ml LPS) produced significant increase in NF-κB p50 subunit mRNA expression in comparison to controls (0 μ*M* PGJ2 and 0 μg/ml LPS). Comparison of NF-κB p50 subunit mRNA in AM exposed to LPS and PGJ2 (10 μ*M* PGJ2+0.1 μg/ml LPS) to LPS alone (0 μ*M* PGJ2+0.1 μg/ml LPS) produced a significant decrease in LPS-induced expression of NF-κB, but levels remained significantly higher than in the control (0 μ*M* PGJ2+0 μg/ml LPS). Thus, PGJ2 inhibited the LPS-induced increase in NF-κB p50 subunit mRNA expression, but did not completely block the LPS-induced rise.

DISCUSSION

PGJ2 was shown to suppress LPS-induced production of TNF-α and NO in mouse AM (Reddy et al., 2004), as well as the production of TNF-α from human AMs (Asada et al., 2004; Bonfield et al., 2003). However, the effect of PGJ2 on inflammatory mediator production from rat AMs has not been reported. Thus, the first major objective of this study was to determine whether PGJ2 inhibited the production of inflammatory mediators from rat AM. To investigate this hypothesis, in vitro studies were conducted using primary rat AM and LPS as an inflammatory agent. Initially, it was determined that 10 μ*M* PGJ2 does inhibit NO production from LPS-exposed primary rat AM at LPS concentrations ranging from 0.1 to 100 μg/ml. Next, a concentration-response curve for PGJ2 inhibition established that PGJ2 concentrations ranging from 1 to 10 μ*M* produced a progressive concentration-dependent decrease in NO production induced by exposure to LPS in the absence of overt toxicity. Furthermore, PGJ2 reduced LPS-induced TNF-α and MIP-2, as well as NO production. When considered together, these observations established that PGJ2 does inhibit LPS-induced inflammatory mediator production by AM.

One possible mechanism that might account for PGJ2 inhibition of LPS-induced inflammatory mediator production would be if PGJ2 interfered with LPS binding to or uptake by AM. To investigate this possibility, a flow cytometric experiment was conducted. These experiments determined that PGJ2 did not interfere with LPS binding to AMs, nor did it alter LPS uptake after binding. This suggested that another mechanism underlying PGJ2 inhibition of LPS-induced inflammatory mediator production was operative in rat AM.

PGJ2 inhibition of LPS-induced production of NO, TNF-α, and MIP-2 was sensitive to time of PGJ2 addition relative to LPS. Specifically, PGJ2 inhibition was strongest when added prior to addition of LPS, and the magnitude of PGJ2 inhibition decreased when added simultaneously or after LPS. Ligand-dependent transcription factors, such as PPAR-γ, were identified as important regulators for macrophage-mediated inflammation (Valledor & Ricote, 2004), and thus represent a possible mechanism that may be present in rat AM. Specifically, a PPAR-γ/co-repressor complex is believed to exist in the cytoplasm of cells (Bishop-Bailey & Hla, 1999; Chinetti et al., 1998). When PGJ2 enters the cell it binds with the PPAR-γ/co-repressor complex, causing the co-repressor to dissociate, which in turn allows interaction with other co-activators, ultimately resulting in translocation to the nucleus (Zhu et al., 1997). Once in the nucleus, the PGJ2/PPAR-γ complex (or complexes) binds to PPAR-response elements in the gene promoter regions, either stimulating or decreasing gene expression. The observations in the present study are consistent with a PPAR-γ dependent mechanism. Specifically, when added prior to LPS, PGJ2 has time to penetrate the cell, activate PPAR-γ, and allows time to translocate to the nucleus where the complex binds to the promoter regions of genes, altering gene expression. When added after LPS exposure, the PGJ2 inhibition is reduced, because gene expression has already been initiated. Thus, the second major objective of this study was to investigate if PGJ2 inhibition of inflammatory mediator production by AM was by a PPAR-γ dependent mechanism.

PPAR-γ is known to be present in both mouse (Reddy et al., 2004) and human (Asada et al., 2004; Bonfield et al., 2003) AM. PPAR-γ was reported to be present in a number of rat cell types, including those present in the central nervous system, epidermis, kidneys, pancreas, heart, spleen, digestive tract, and genital system, but not the lung or AM in particular (Braissant

et al., 1996). In order for the hypothesis that PGJ2 inhibition of inflammatory mediator production by AM is by a PPAR-γ-dependent mechanism to be a viable possibility, it was necessary to first establish the presence of PPAR-γ in rat AMs. RT-PCR analyses demonstrated PPAR-γ mRNA expression in unstimulated rat AM, and Western blot analysis was positive for PPAR-γ protein in naive primary rat BAL cells. Thus, the hypothesis that PGJ2 inhibition of inflammatory mediator production may be via a PPAR-γ dependent mechanism is a viable possibility.

To test the hypothesis that PGJ2 inhibition is via PPAR-γ dependent mechanism, the effects of CG and GW9662 on LPS-induced expression of NO were determined. Like PGJ2, CG is a PPAR-γ ligand, but unlike PGJ2, CG did not inhibit LPS-induced expression of NO from rat AM. Furthermore, GW9662, a PPAR-γ antagonist, exerted no effect on PGJ2-mediated inhibition of LPS-induced NOx production. In addition, LPS exposure produced a significant reduction in PPAR-γ mRNA, and PGJ2 did not prevent this LPS-induced effect. Similar results were reported for microglial cells, where LPS was shown to also decrease expression of PPAR-γ (Bernardo et al., 2000). However, unlike rat AM, PGJ2 prevented the LPS-induced reduction in PPAR-γ expression in microglial cells (Bernardo et al., 2000). Taken together, these biochemical and molecular data indicate that PGJ2 inhibition of LPS-induced inflammatory mediator production is not PPAR-γ dependent in primary rat AM. Consequently, a PPAR-γ-independent mechanism for PGJ2 inhibition of LPS-induced inflammatory mediator production is operative.

Indeed, PGJ2 was reported to exert effects on cells that do not have PPAR-γ (Chawla et al., 2001), and thus its effects may be mediated via PPAR-γ-independent mechanisms. NF-κB was suggested as a possible target through which PGJ2 might also regulate inflammatory responses (Scher & Pillinger, 2005). In resting cells, NF-κB is located in the cytoplasm in an inactive form and is associated one of several inhibitory proteins. Activation of the NF-κB signaling cascade results in degradation of the inhibitory protein, allowing translocation into the nucleus. Once in the nucleus, NF-κB binds with promoter regions of genes, stimulating their expression (Chen, et al 2002).

It was previously reported that after exposure to LPS in vitro, NF-κB activation with concomitant NO production occurs in primary rat AM (Yu et al., 1997). In this study, it was also found that in vitro LPS exposure of primary rat AM induces (1) a significant increase in NO production, as well as TNF-α and MIP-2, and (2) increased expression of NF-κB p50 subunit mRNA. Furthermore, it was determined that PGJ2 significantly reduces the LPS-induced increase in NF-κB mRNA and inflammatory mediators, but neither returned to control levels. This indicates that PGJ2 effects on LPS-induced inflammatory mediator production in rat AM are mediated via altering expression of NF-κB p50 subunit mRNA. This mechanism of PGJ2 action differs from reports from other labs, which reported that PGJ2 altered NF-κB activity by inducing synthesis of IκB (Castrillo et al., 2000), binding to and inhibiting the action of IκB kinase (Rossi et al., 2000), or by inhibiting NF-κB binding to DNA (Straus et al., 2000).

The experiments in this study established that PGJ2 inhibits the expression of inflammatory mediators in rat AMs in a concentration- and time-dependent manner. Biochemical and molecular studies suggested that the mechanism underlying PGJ2 inhibition was not through a PPAR-γ dependent mechanism, but rather a NF-κB mechanism. Interestingly, a similar mechanism may be operative in human AM. Specifically, in comparison to healthy controls, patients with sarcoidosis, a disease characterized by ongoing inflammation, show decreased expression of PPAR-γ mRNA, and increased activation of NF-κB (Culver et al., 2004). This is similar to the effect of LPS on rat AM, i.e., LPS decreased expression of PPAR-γ and increased expression of NF-κB mRNAs.

In conclusion, the in vivo physiological relevance of PGJ2 needs to be considered. Evidence for PGJ2 synthesis in vivo comes from a report that detected PGJ2 in human urine (Hirata et al., 1988) and esterified in rat tissue membrane lipids (Chen et al., 1999). Furthermore, exogenous administration of PGD2 to cynomolgus monkeys induces large increases in urine PGJ2 concentrations, indicating that PGD2 is the in vivo precursor of PGJ2 (Hirata et al., 1988). The dependence of PGJ2 synthesis on PGD2 suggests that formation of PGJ2 adducts is delayed relative to synthesis of other prostaglandins, and that PGJ2 may participate in resolution of prostaglandin-mediated inflammation. This hypothesis was supported in a rat study which used a carrageenin-induced inflammatory model. In this study, it was determined that cyclooxygenase-2 (COX-2) was proinflammatory due to PGE2 production during the development of inflammation, but anti-inflammatory during resolution due to increased PGJ2 production (Gilroy et al., 1999). These in vivo studies, in conjunction with the findings in the present in vitro study, suggest that PGJ2 may regulate AM inflammatory mediator expression and production in vivo.

REFERENCES

Asada, K., Sasaki, S., Suda T.,, Chida, K., and Nakamura, H. 2004. Anti-inflammatory roles of peroxisome proliferator-activated receptor gamma in human alveolar macrophages. *Am. J. Respir. Crit. Care Med.* 169:195–200.

Azuma, Y., Shinohara, M., Wang, P. L., and Ohura, K. 2001. 15-Deoxy-delta(12,14)-prostaglandin J(2) inhibits IL-10 and IL-12 production by macrophages. *Biochem. Biophys. Res. Commun.* 283:344–346.

Bernardo, A., Levi, G., and Minghetti, L. 2000. Role of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and its natural ligand 15-deoxy-Delta12, 14-prostaglandin J2 in the regulation of microglial functions. *Eur. J. Neurosci.* 12:2215–2223.

Bishop-Bailey, D., and Hla, T. 1999. Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy-δ-12,14-prostaglandin J2. *J. Biol. Chem.* 274:17042–17048.

Bonfield, T. L., Farver, C. F., Barna, B. P., Malur, A., Abraham, S., Raychaudhuri B.,, Kavuru, M. S., and Thomassen, M. J. 2003. Peroxisome proliferator-activated receptor-gamma is deficient in alveolar macrophages from patients with alveolar proteinosis. *Am. J. Respir. Cell Mol. Biol.* 29:677–682.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. 1996. Differential expression of peroxisome proliferator-activated receptors (PPARs): Tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* 137:354–366.

Castrillo, A., Diaz-Guerra, M. J., Hortelano, S., Martin-Sanz P., and Bosca, L. 2000. Inhibition of IkappaB kinase and IkappaB phosphorylation by 15-deoxy-delta(12,14)-prostaglandin J(2) in activated murine macrophages. *Mol. Cell Biol.* 20:1692–1698.

Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., and Evans, R. M. 2001. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat. Med.* 7:48–52.

Chen, Y., Morrow, J. D., and Roberts, L. J. 1999. Formation of reactive cyclopentenone compounds in vivo as products of the isoprostane pathway. *J. Biol. Chem.* 274:10863–10868.

Chen, F., L. Demers, M., and Shi, X. 2002. Upstream signal transduction of NF-kappaB activation. *Curr. Drug Targets Inflamm. Allergy* 1:137–149.

Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J., and Staels, B. 1998. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. *J. Biol. Chem*. 273:25573–25580.

Culver, D. A., Barna, B. P., Raychaudhuri, B., Bonfield, T. L., Abraham, S., Malur, A., Farver, C. F., Kavuru, M. S., and Thomassen, M. J. 2004. Peroxisome proliferator-activated receptor gamma activity is deficient in alveolar macrophages in pulmonary sarcoidosis. *Am. J. Respir. Cell Mol. Biol*. 30:1–5.

Gilroy, D. W., Colville-Nash, P. R., Willis, D., Chivers, J., Paul-Clark, M. J., and Willoughby, D. A. 1999. Inducible cyclooxygenase may have anti-inflammatory properties. *Nat. Med*. 5:698–701.

Hirata, Y., Hayashi, H., Ito, S., Kikawa, Y., Ishibashi, M., Sudo, M., Miyazaki, H., Fukushima, M., Narumiya, S., and Hayaishi, O. 1988. Occurrence of 9-deoxy-Δ^9, Δ^{12}-13,14-dihydroprostaglandin D_2 in human urine. *J. Biol. Chem*. 263:16619–16625.

Kawahito, Y., Kondo, M., Tsubouchi, Y., Hashiramoto, A., Bishop-Bailey, D., Inoue, K., Kohno, M., Yamada, R., Hla, T., and Sano, H. 2000. 15-Deoxy-delta(12,14)-PGJ(2) induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. *J. Clin. Invest*. 106:189–197.

Landreth, G. E., and Heneka, M. T. 2001. Anti-inflammatory actions of peroxisome proliferator-activated receptor gamma agonists in Alzheimer's disease. *Neurobiol. Aging* 22:937–944.

Mochizuki, M., Ishii, Y., Itoh, K., Iizuka, T., Morishima, Y., Kimura, T., Kiwamoto, T., Matsuno, Y., Hegab, A. E., Nomura, A., Sakamoto, T., Uchida, K., Yamamoto, M., and Sekizawa, K. 2005. Role of 15-deoxy delta(12,14) prostaglandin J2 and Nrf2 pathways in protection against acute lung injury. *Am. J. Respir. Crit. Care Med*. 171:1260–1266.

Narumiya, S., Sugimoto, Y., and Ushikubi, F. 1999. Prostanoid receptors: structures, properties, and functions. *Physiol. Rev*. 79:1193–1226.

Park, G. Y., and Christman, J. W. 2006. Nuclear factor kappa B is a promising therapeutic target in inflammatory lung disease. *Curr. Drug Targets* 7:661–668.

Porter, D. W., Millecchia, L., Robinson, V. A., Hubbs, A., Willard, P., Pack, D., Ramsey, D., McLaurin, J., Khan, A., Landsittel, D., Teass, A., and Castranova, V. 2002. Enhanced nitric oxide and reactive oxygen species production and damage after inhalation of silica. *Am. J. Physiol. Lung Cell Mol. Physiol.* 283:L485–L493.

Reddy, R. C., Keshamouni, V. G., Jaigirdar, S. H., Zeng, X., Leff, T., Thannickal, V. J., and T. J. Standiford, 2004. Deactivation of murine alveolar macrophages by peroxisome proliferator-activated receptor-gamma ligands. *Am. J. Physiol. Lung Cell Mol Physiol.* 286:L613–L619.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391:79–82.

Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. G. 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 403:103–108.

Scher, J. U., and Pillinger, M. H. 2005. 15d-PGJ2: The anti-inflammatory prostaglandin? *Clin. Immunol*. 114:100–109.

Straus, D. S., Pascual, G., Li, M., Welch, J. S., Ricote, M., Hsiang, C. H.,, Sengchanthalangsy, L. L., Ghosh, G., and Glass, C. K. 2000. 15-Deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proc. Natl. Acad. Sci. USA* 97:4844–4849.

Su, C. G., Wen, X., Bailey, S. T., Jiang, W., Rangwala, S. M., Keilbaugh, S. A., Flanigan, A., Murthy, S., Lazar, M. A., and Wu, G. D. 1999. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. *J. Clin. Invest*. 104:383–389.

Valledor, A. F., and Ricote, M. 2004. Nuclear receptor signaling in macrophages. *Biochem. Pharmacol*. 67:201–212.

Yu, S. M., Wu, J. F., Lin, T. L., and Kuo, S. C. 1997. Inhibition of nitric oxide synthase expression by PPM-18, a novel anti-inflammatory agent, in vitro and in vivo. *Biochem. J*. 328:363–369.

Zhu, Y., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. 1997. Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. *J. Biol. Chem*. 272:25500–25506.