Inhibition of IFN-γ-Mediated Inducible Nitric Oxide Synthase Induction by the Peroxisome Proliferator-Activated Receptor γ Agonist, 15-Deoxy-Δ12,14-Prostaglandin J2, Involves Inhibition of the Upstream Janus Kinase/STAT1 Signaling Pathway

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Inhibition of IFN-γ-Mediated Inducible Nitric Oxide Synthase Induction by the Peroxisome Proliferator-Activated Receptor γ Agonist, 15-Deoxy-Δ^{12,14}-Prostaglandin J₂, Involves Inhibition of the Upstream Janus Kinase/STAT1 Signaling Pathway

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Peroxisome proliferator-activated receptor γ (PPARγ) ligands have been reported to exert anti-inflammatory activities in macrophages by competition for transcriptional coactivators with some transcriptional factors, including NF-κB. In the present study the influence of PPARγ activators on IFN-γ-elicited macrophage stimulation and signaling cascades was investigated. The results show that IFN-γ-induced inducible NO synthase (iNOS) gene transcription, iNOS protein induction, and NO production are more sensitive to inhibition by 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15dPGJ₂) than by the other two PPARγ agonists, GW1929 and ciglitazone. Delayed addition of 15dPGJ₂ for 2 h resulted in reduced inhibition, suggesting action by 15dPGJ₂ on the upstream signaling cascades. Immunoblotting, DNA binding, and reporter gene assays consistently revealed the inhibitory ability of 15dPGJ₂, but not GW1929 or ciglitazone, on IFN-γ-elicited signaling cascades, including tyrosine phosphorylation of Janus tyrosine protein kinase 2 and STAT1, DNA binding, and IFN regulatory factor-1 trans-activation of STAT1. These effects of 15dPGJ₂ were not abrogated by the PPARγ antagonist, bisphenol A diglycidyl ether, indicating the PPARγ-independent actions. 15dPGJ₂ also attenuated IL-6-induced tyrosine phosphorylation of STAT1 and STAT3 in Hep3B hepatoma cells. Consistent with the inhibitory effect of reactive oxygen species on STAT1 signaling, STAT1 inhibition by 15dPGJ₂ was abrogated by N-acetylcysteine, glutathione, superoxide dismutase, and catalase. Furthermore, 15dPGJ₂-induced inhibition of STAT1 phosphorylation and NO production still occurred in the presence of peroxovanadate, ruling out the action mechanism of 15dPGJ₂ on tyrosine phosphatase. Taken together, for the first time in this study we demonstrate that 15dPGJ₂ can inhibit cytokine-stimulated Janus kinase 2-STAT signaling through a PPARγ-independent, reactive oxygen species-dependent mechanism. These data provide a novel molecular mechanism of iNOS inhibition by 15dPGJ₂, and confirm its physiological role in anti-inflammation.


Interferon-γ, the prototypical Th1 cell cytokine, can interfere with viral replication and affect many cell functions (1–3). The pleiotropic biological effects of IFN-γ rely on its actions in regulating gene transcription. The intracellular signaling pathway triggered by IFN-γ receptors involves the rapid and direct activation of Janus tyrosine protein kinases (JAKs)²-STAT signaling pathways (2, 4–7). This pathway involves cell membrane-localized JAK1 and JAK2 for tyrosine phosphorylation and activation of latent cytoplasmic STAT1. After activation by JAK through the rapid tyrosine phosphorylation of Y701, STAT1 is released from the receptor, forms a homodimer, and is translocated to the nucleus, where it binds to IFN-γ-activated sequence (GAS) elements (7). Numerous biological actions of IFN-γ have been demonstrated to be dependent on STAT1 signal transduction pathway-associated gene products. One of the STAT1-induced gene products is IFN regulatory factor-1 (IRF-1), which is a transcription factor and recognizes the sequence called IFN-stimulated regulatory elements (ISRE). IRF-1 is strongly inducible after IFN-γ stimulation and participates in the transcription of many IFN-regulated secondary genes whose promoters contain ISRE (8, 9).

Macrophages, functioning as a key effector of cell populations in innate and adaptive immune responses, are known to be the targets by which IFN-γ accelerates Th1 cell development and kills microbial targets. For this purpose, IFN-γ acts cooperatively with LPS, a component of the cell wall of Gram-negative bacteria, to induce many of the genes involved in inflammation (10). The molecular mechanisms underlying synergistic gene induction involve the cooperative activation of transcription factors. For example, enhancement of NO production from macrophages by simultaneous co-incubation with both stimuli results from the synergistic functions of IRF-1 and STAT1 with NF-κB activated by LPS in transcription of the gene encoding the inducible form of NO synthase (iNOS) (9, 11–13). The well-characterized 1.7-kb fragment of the 5’-flanking region of the murine iNOS gene contains at least 10 copies of IFN-γ response elements (IREs), three copies of the GAS, two copies of the ISRE, and two copies of NF-κB (12, 14). Substantial evidence has demonstrated that the transcriptional induction of iNOS by IFN-γ depends on JAK-STAT activation (11, 15).
Peroxisome proliferator-activated receptors (PPARs), transcription factors belonging to the nuclear receptor superfamily, have been reported to play a major role in regulating a number of genes implicated in inflammation (16, 17). The three different PPAR subtypes identified to date, PPARα, PPARβ/δ, and PPARγ, can regulate gene expression by binding with the retinoid receptor, RXR, as a heterodimeric partner to specific DNA sequence elements termed PPAR response elements (PPREs) (18). With respect to immunoregulation, PPARγ activation has been shown to repress LPS-, phorbol ester-, and cytokine-induced activation of a number of inflammatory genes, such as iNOS, gelatinase B, scavenger receptor A, cyclooxygenase-2, TNF-α, IL-1β, IL-6, and chemokines (16, 19–23). In addition, PPARγ activators were reported to inhibit IFN-γ-induced mRNA and protein expression of CXC chemokines (20). These actions of PPARγ agonists were proven through the PPARγ-dependent repression of activities of the transcription factors, NF-κB, AP-1, STAT1, and Sp1. This transcription repression involves competition for limited amounts of general transcription coactivators (24, 25). To date, limited information is available for the interaction between the actions of PPARγ and IFN-γ. Except for acting on the transcription repressor, PPARγ, in antagonizing the activity of STAT1 (16, 20), it is not known whether additional target sites of PPARγ agonists are involved in the impairment of IFN-γ’s action. Here, we compare the inhibition by three PPARγ agonists, 15-deoxy-Dα,β-21,24-prostaglandin J2 (15dPGJ2), GW1929, and ciglitazone, on IFN-γ induction of NO production and investigate the mechanisms underlying this regulation.

Materials and Methods

Materials

DMEM, FBS, penicillin, and streptomycin were obtained from Life Technologies (Grand Island, NY). Rabbit polyclonal Ab against INOS was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal Abs against active (phosphorylated) STAT1 (Y701) and STAT3 (Y705) were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal Ab against JAK2 (Y1007, Y1008) was purchased from Upstate Biotechnology (Lake Placid, NY). IFN-γ and IL-6 were purchased from R&D (Minneapolis, MN). 15dPGJ2 was obtained from Cayman (Ann Arbor, MI). GW1929 was purchased from Glaxo Wellcome. BADGE (bisphenol A diglycidyl ether) was obtained from Tocris Cookson (Avonmouth, U.K.). PD98059, PP2, genistein, AG490, SB203580, Ro31–8220, and GF109203X were purchased from Calbiochem (San Diego, CA). α-[32P]ATP (3000 Ci/mmol), HRP-coupled anti-mouse and anti-rabbit Abs, and the ECL detection agent were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Rabbit polyclonal Abs specific for iNOS, STAT1, STAT3, JAK2, PPARα, PPARγ, and protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The luciferase reporter construct of the human IRF-1 gene promoter (−1.3 kbp) containing one GAS element was provided by Dr. Y. Ohmori (Cleveland Clinic Foundation, Cleveland, OH). PD98059, PP2, genistein, AG490, SB203580, Ro31–8220, and GF109203X were purchased from Calbiochem (San Diego, CA). α-[32P]ATP (3000 Ci/mmol), HRP-coupled anti-mouse and anti-rabbit Abs, and the ECL detection agent were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Rabbit polyclonal Abs specific for iNOS, STAT1, STAT3, JAK2, PPARα, PPARγ, and protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The luciferase reporter construct of the human IRF-1 gene promoter (−1.3 kbp) containing one GAS element was provided by Dr. Y. Ohmori (Cleveland Clinic Foundation, Cleveland, OH). The luciferase reporter plasmid containing the (PPRE consensus) × 2 thymidine kinase promoter was provided by Dr. W. Wahl (University of Lausanne, Lausanne, Switzerland). The oligonucleotide of sS-inducible element (SIE) (5′-ATCGTTCATTTCCCGTAAATCCCTA-3′) was synthesized with a TPS250 DNA synthesizer (Cruachem, Herndon, VA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All materials for SDS-PAGE were obtained from Bio-Rad (Hercules, CA).

Cell culture

Murine RAW264.7 macrophages, human colon adenocarcinoma HT-29, and Hep3B hepatoma cell lines obtained from American Type Culture Collection (Manassas, VA) were grown at 37°C in 5% CO2 using DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Nitrite assay

Nitrite production, as an assay of NO release, was measured. Accumulation of nitrite in the medium was determined by a colorimetric assay with Griess reagent. Cells were treated with vehicle, LPS, IFN-γ, and/or PPARγ agonists at the indicated concentrations for different intervals. Aliquots of conditioned medium were mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine in 5% phosphoric acid). Nitrite concentrations were determined by comparison with the OD550 using standard solutions of sodium nitrite prepared in cell culture medium. Each experiment was performed in duplicate and repeated at least three times.

Western blot analysis

After incubation with vehicle, IFN-γ, or PPARγ agonist, cells were washed twice in ice-cold PBS and then solubilized in buffer containing 1% Triton X-100, 125 mM NaCl, 1 mM MgCl2, 25 mM β-glycerophosphate, 50 mM NaF, 100 μM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 20 mM Tris-HCl, pH 7.5. Samples of equal amounts of protein (80 μg) were separated through SDS-10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose paper by semidry electrophoretic transfer. Membranes were blocked in TBS containing 0.1% Tween 20 (TBST) and 5% nonfat milk for 1 h. Proteins were visualized by specific primary Abs, followed by peroxidase-labeled secondary Abs. Immunoreactivity was detected by ECL reagents following the manufacturer’s instructions, with exposure to x-ray film and quantification by densitometer. To examine the stability of the INOS protein, cells were initially treated with IFN-γ overnight, followed by washout and addition of anisomycin (3 μg/ml) and the indicated drugs for different periods.

RT-PCR

To amplify iNOS mRNA, the specific primers for RT-PCR analysis were synthesized. Macrophages treated with the indicated agents were homogenized in 1 ml of RNAzol B reagent (Life Technologies), and total RNA was extracted by an acid guanidinium thiocyanate-phenol-chloroform extraction. RT was performed using a StrataScript RT-PCR kit, and 10 μg of total RNA was reverse transcribed to cDNA following the manufacturer’s recommended procedures. RT-generated cDNA encoding iNOS and β-actin genes was amplified using PCR. The oligonucleotide primers used correspond to mouse macrophage iNOS (5′-CCC TTC CTC AGT TTT GTC TGG CAG CAG C-3′ and 5′-GGG TGT CAG AGC CTC GTG GCT TTG G-3′) and mouse β-actin (5′-GAC TAC CTC ATG AAG ATC CT-3′ and 5′-CCA CAT CTG CCT GAA GGT GG-3′). PCR was performed in a final volume of 50 μl containing Taq DNA polymerase buffer, all four dNTPs, oligonucleotide primers, Taq DNA polymerase, and RT products. After initial denaturing for 2 min at 94°C, 35 cycles of amplification (94°C for 45 s, 65°C for 45 s, and 72°C for 2 min) were performed, followed by a 10-min extension at 72°C. PCR products were analyzed on 2% agarose gels. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared as previously described (26) and incubated in containing mixtures of 2.5 μg poly(dI-dC), 20,000 rpm of the 32P-labeled STAT binding sites (for STAT1, 3 μM Tris-HCl, pH 7.5, 1.1 μM AcTAC ATG ATC ATC CTT G-3′, and 3′-GCC CAT CTT GCA CCTGAG GCT GTT TTG G-3′) and mouse β-actin (5′-GAC TAC CTC ATG AAG ATC CT-3′ and 5′-CCA CAT CTG CCT GAA GGT GG-3′). PCR was performed in a final volume of 50 μl containing Taq DNA polymerase buffer, all four dNTPs, oligonucleotide primers, Taq DNA polymerase, and RT products. After initial denaturing for 2 min at 94°C, 35 cycles of amplification (94°C for 45 s, 65°C for 45 s, and 72°C for 2 min) were performed, followed by a 10-min extension at 72°C. PCR products were analyzed on 2% agarose gels. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Transfection and reporter gene assay

Using electroporation (280 V, 1070 μF, 0.4-ms time constant), RAW264.7 cells (2 × 105 cells/cuvette) were cotransfected with 1 μg IRF-1 promoter plasmid and 1 μg β-galactosidase expression vector. In transfection assays of HT-29 cells, 2 × 105 cells seeded into 12-well plates were transfected on the following day by Lipofectamine 2000 (LF2000; Life Technologies), Premix DNA with Opti-MEM, and LF2000 with Opti-MEM, respectively, for 5 min. Then the mixture was incubated for 25 min at room temperature and added to each well. After a 24-h incubation, transfection was complete, and cells were incubated with the indicated agents. After a 14-h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 50 μl of reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30 s. Aliquots of cell lysates (5 μl) containing equal amounts of protein (10–20 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate (Promega) was added to all samples, and luminescence was measured in a microplate luminometer (Meridian, CT). The luciferase activity value was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector (pCR3lacZ; Amersham Pharmacia Biotech, Uppsala, Sweden). The level of induction of luciferase activity was determined as a ratio compared with cells with no stimulation.
Statistical evaluation

Values were expressed as the mean ± SEM of at least three experiments, which were performed in duplicate. ANOVA was used to assess the statistical significance of the differences, and *p < 0.05 was considered statistically significant.

Results

15dPGJ2 attenuated IFN-γ-induced NO formation at the transcriptional level

Following stimulation with LPS (0.1–1 μg/ml; Fig. 1A) or IFN-γ (1–10 ng/ml; Fig. 1B) for 24 h, a large amount of NO was detected in the culture medium of RAW264.7 macrophages. The stimulatory effects of LPS and IFN-γ exhibited a concentration dependency. When each of the three PPARγ agonists, ciglitazone (10 μM), GW1929 (10 μM), and 15dPGJ2 (0.3–10 μM), was coincubated with stimulus, the NO responses of LPS and IFN-γ were significantly inhibited (Fig. 1, A and B). In terms of the inhibitory extents and potencies, 15dPGJ2 was the most potent and efficacious PPARγ agonist in this study. 15dPGJ2 displayed IC_{50} values of 0.3, 1, and 1 μM with respect to LPS stimulation at 0.1, 0.3, and 1 μg/ml, respectively, and of 0.5, 3, and 4 μM with respect to IFN-γ stimulation at 1, 3, and 10 ng/ml, respectively. In addition, the maximal inhibitory effect of 15dPGJ2 reached 100% when a moderate increase in NO release of <20 μM was induced by 0.1 μg/ml of LPS or 1–10 ng/ml of IFN-γ. On the contrary, regardless of whether LPS or IFN-γ treatment was used at different concentrations, 10 μM ciglitazone and GW1929 caused a moderate inhibition of NO formation, which ranged from 20–40%. The greater extent of NO inhibition by 15dPGJ2 than the other PPARγ agonists implies that a PPARγ-independent mechanism contributes to 15dPGJ2’s action. Consistent with these results, iNOS protein expression was abrogated by 15dPGJ2, but was only partially reduced by ciglitazone and GW1929 (Fig. 1C). At the concentrations tested, each PPARγ agonist plus LPS (1 μg/ml) or IFN-γ (10 ng/ml) had no effect on cell viability (data not shown).

Previous studies have proven that PPARγ agonists can transcriptionally inhibit iNOS gene expression by interrupting the essential trans-activation driven by nuclear NF-κB and STAT1 (16). Furthermore, the more potent inhibition by 15dPGJ2 than that by ciglitazone or GW1929 on the LPS response can undoubtedly be explained by its direct interaction and inhibition of the upstream signaling kinase for NF-κB activation, i.e., IκB kinase (IKK) (27, 28). Despite such nonspecific action of 15dPGJ2, this NF-κB inhibition mechanism cannot be applied to IFN-γ sensitivity. In view of the fact that IFN-γ itself cannot cause NF-κB activation in RAW264.7 macrophages (data not shown), it is of great interest to further explore additional and possibly PPARγ-independent mechanisms of 15dPGJ2, which allow 15dPGJ2 to elicit higher inhibition of IFN-γ stimulation. Before answering this question, we asked whether 15dPGJ2 inhibition of iNOS-derived NO release following IFN-γ stimulation occurs at the transcriptional level. To address this question, we first determined the iNOS mRNA level. As shown in Fig. 2A, the IFN-γ-induced iNOS mRNA level was concentration-dependently reduced in the presence of 15dPGJ2. At 10 μM, 15dPGJ2 abolished mRNA expression, while ciglitazone and GW1929 only partially inhibited it. Second, to understand the effect on iNOS protein stability, we treated PPARγ agonists after iNOS induction by IFN-γ. Under the condition of anisomycin-blocking gene transcription, the iNOS protein was gradually degraded, and this turnover rate was not significantly changed by the presence of any of the PPARγ agonists (Fig. 2B). Third, we measured the time-dependent effect of 15dPGJ2. As shown in Fig. 2C, maximal inhibition in the presence of 15dPGJ2 was obtained upon simultaneous treatment of 15dPGJ2 with IFN-γ. Delayed treatment of 15dPGJ2 for 2–6 h resulted in partial and consistent inhibition of 25–40%, comparable to the inhibitory effects of ciglitazone and GW1929. These results suggest that 15dPGJ2 might interfere with IFN-γ-induced iNOS gene expression through at least two mechanisms. The first mechanism is suppression of STAT1 trans-activation, as previously demonstrated, through competition with transcription cofactors by PPARγ activation (24). The second mechanism might be an uncoupling of the upstream signaling induced by IFN-γ through a PPARγ-independent pathway.

15dPGJ2 inhibition of IFN-γ-induced STAT1 tyrosine phosphorylation and DNA-binding activity

Previous reports have indicated that the expression of iNOS is regulated coordinately by the transcription factors NF-κB, AP-1, and STAT1. To address the PPARγ-independent mechanism of 15dPGJ2 on IFN-γ signaling, we first used pharmacological agents

FIGURE 1. PPARγ agonists concentration-dependently inhibited LPS- and IFN-γ-induced NO production in RAW264.7 macrophages. RAW264.7 cells were treated with vehicle, 15dPGJ2, GW1929, or ciglitazone at the concentrations indicated for 20 min, followed by stimulation with LPS (0.1–1 μg/ml; A) or IFN-γ (1–10 ng/ml; B) for 24 h. The stable NO metabolite nitrite present in the medium was analyzed by the Griess method. The results shown are the mean ± SEM from three to five independent experiments, each performed in duplicate. *p < 0.05, significant inhibition with PPARγ agonist treatment. C, Cells were treated with vehicle, IFN-γ (10 ng/ml), and/or PPARγ agonists at the concentrations indicated for 24 h, followed by iNOS protein measurement with immunoblotting. The data in parentheses indicate the percentage of iNOS induction compared with the control response without PPARγ agonist treatment.
at concentrations specific for each target to confirm the essential role of JAK-STAT1 in IFN-\(\gamma\)’s action. As shown in Fig. 3A, we found that the NO response of IFN-\(\gamma\) was significantly inhibited by the nonselective tyrosine kinase inhibitor genistein (30 \(\mu\)M) and the selective JAK2 inhibitor AG490 (30 \(\mu\)M) (29). However, inhibitors of NF-\(\kappa\)B (10 \(\mu\)M pyrrolidine dithiocarbamate (PDTC), protein kinase C (PKC; 1 \(\mu\)M Ro 31–8220, and 3 \(\mu\)M GF109203X), mitogen-activated protein/ERK kinase (MEK; 50 \(\mu\)M PD98059), p38 mitogen-activated protein kinase (MAPK; 3 \(\mu\)M SB203580), and Src (1 \(\mu\)M PP2) did not affect the IFN-\(\gamma\)-induced NO increase. These results suggest that the activation of the JAK-STAT1 signaling pathway is sufficient in macrophages to mediate IFN-\(\gamma\)-induced iNOS gene expression.

Next, we investigated the effects of PPAR\(\gamma\) agonists on the DNA binding activity of STAT1 after IFN-\(\gamma\) stimulation in macrophages. Nuclear extracts of macrophages treated with IFN-\(\gamma\) were subjected to EMSA using SIE as a probe. As shown in Fig. 3B, SIE binding was clearly induced after 30 min of stimulation with IFN-\(\gamma\). Gel retardation analysis by mixing the STAT1 Ab

FIGURE 2. 15dPGJ\(_2\) inhibition of IFN-\(\gamma\)-induced iNOS gene transcription. A, Cells were treated with vehicle, IFN-\(\gamma\) (10 ng/ml), and/or PPAR\(\gamma\) agonists at the concentrations indicated for 12 h, followed by iNOS mRNA measurement with RT-PCR. The \(\beta\)-actin mRNA level was considered the internal control. The data in parentheses indicate the percentage of iNOS induction compared with the control response without PPAR\(\gamma\) agonist treatment. B, In iNOS-expressing cells following IFN-\(\gamma\) (10 ng/ml) treatment for 24 h, IFN-\(\gamma\) was washed out, and anisomycin (3 \(\mu\)g/ml) was included together with PPAR\(\gamma\) agonists for 4, 8, 12, or 16 h. At these time points, cell lysates were subjected to SDS-PAGE, and iNOS protein was determined by immunoblotting, followed by densitometry. The results are representative of three independent experiments with similar results. C, 15dPGJ\(_2\) (3 \(\mu\)M) or vehicle was added simultaneously with or several hours after IFN-\(\gamma\) (3 or 10 ng/ml) stimulation in RAW264.7 cells. Twenty-four hours after IFN-\(\gamma\)-treatment, the nitrite level in the medium was assayed. Basal level of nitrite. Data are the mean \(\pm\) SEM from at least three independent experiments, each performed in duplicate. *, \(p < 0.05\), significant inhibition with PPAR\(\gamma\) agonist treatment.

FIGURE 3. The JAK/STAT signaling pathway involved in iNOS induction by IFN-\(\gamma\) is inhibited by 15dPGJ\(_2\). A, Cells were pretreated with vehicle, genistein (30 \(\mu\)M), AG490 (30 \(\mu\)M), PP2 (1 \(\mu\)M), PDTC (10 \(\mu\)M), Ro 31–8220 (1 \(\mu\)M), GF109203X (3 \(\mu\)M), PD98059 (50 \(\mu\)M), or SB203580 (3 \(\mu\)M) for 20 min before the addition of 10 ng/ml IFN-\(\gamma\). Data are the mean \(\pm\) SEM from three independent experiments, each performed in duplicate. *, \(p < 0.05\), significant inhibition.

B, Cells were treated with 10 ng/ml IFN-\(\gamma\) with or without 15dPGJ\(_2\) (3–30 \(\mu\)M), ciglitazone (10 \(\mu\)M), or GW1929 (10 \(\mu\)M) for 30 min. Equivalent nuclear extracts prepared from cells were assayed for binding activity with a specific oligonucleotide containing binding sequences for STAT. In one experiment the specific Ab for STAT1 was included in the binding solution. The results are representative of three independent experiments.
verified that the nuclear complex following IFN-γ stimulation was the STAT1 homodimer. Results revealed that STAT1 activation was inhibited by 15dPGJ₂ (3–30 μM) in a concentration-dependent manner, while ciglitazone and GW1929, each at 10 μM, did not change IFN-γ stimulation.

Since Tyr²⁷⁰¹ phosphorylation of STAT1 following IFN-γ stimulation is associated with IFN-γ priming for iNOS expression (15), we then examined the effects of 15dPGJ₂ on STAT1 phosphorylation. As shown in Fig. 4A, in RAW264.7 macrophages treated with IFN-γ, a dramatic and concentration-dependent STAT1 phosphorylation was observed. 15dPGJ₂, GW1929, and ciglitazone individually did not stimulate STAT1 phosphorylation (data not shown), while only 15dPGJ₂ could significantly attenuate IFN-γ-induced STAT1 phosphorylation. The effect of 15dPGJ₂ displayed concentration-dependency (Fig. 4B). After Tyr²⁷⁰¹ phosphorylation, STAT1 formed a homodimer and was translocated to the nucleus. Immunoblot analysis of nuclear proteins consistently indicated that the nuclear level of phosphorylated STAT1 following IFN-γ treatment was also diminished by the presence of 15dPGJ₂ (3–30 μM; Fig. 4C).

Inhibition of STAT1-stimulated IRF-1 expression by 15dPGJ₂ occurs through two mechanisms

To further distinguish and confirm the contribution of PPARγ-dependent action (i.e., as to PPARγ activation) and PPARγ-independent action (i.e., as to STAT1 inhibition) of 15dPGJ₂, ciglitazone, and GW1929 in regulating iNOS transcription, we measured their individual actions on two reporter systems, which are driven, respectively, by PPARγ/PPRE and STAT1/GAS. To achieve high transfection and expression efficiencies in this experiment, HT-29 cells were used for transient transfection of PPARγ and the IRF-1 reporter gene. Before this experiment we analyzed the expression patterns of PPAR isoforms in RAW264.7 macrophages and HT-29 cells. Immunoblotting data revealed that PPARγ was the predominant isoform, and that its level was comparable in both cell types. In contrast, PPARα and PPARδ were hardly detected in RAW264.7 macrophages, while they were also expressed in HT-29 cells in much lower amounts than PPARγ (Fig. 5A). In the PPARγ-dependent luciferase assay, we found that the potency order of these three PPARγ agonists was GW1929 > 15dPGJ₂ = ciglitazone (Fig. 5B). A lower concentration of GW1929 (0.1 μM) began to activate PPARγ, while higher concentrations of ciglitazone (10 μM) and 15dPGJ₂ (3 μM) were required to achieve comparable stimulation. This potency order is quite consistent with those of previous studies examining their binding affinities and trans-activation abilities on PPARγ (19, 30, 31).

The IRF-1 reporter plasmid containing a GAS-binding element was transfected to HT-29 cells and RAW264.7 macrophages and was used to verify the unique action of 15dPGJ₂ on STAT1 signaling. Fig. 5C shows that GW1929 and ciglitazone could inhibit IFN-γ-induced IRF-1 expression by ~30% at 10 μM. In contrast, compared with this moderate inhibition by ciglitazone and GW1929, 15dPGJ₂ at concentrations >0.3 μM, under a condition that 15dPGJ₂ cannot induce PPARγ activation, was sufficient to

FIGURE 4. Inhibition of IFN-γ-induced STAT1 phosphorylation at tyrosine 701 by 15dPGJ₂. Confluent cells were treated with vehicle, IFN-γ (1, 3, or 10 ng/ml), or PPARγ agonists at the concentrations indicated for 15 min unless otherwise indicated. Equivalent protein amounts of total cell lysates (A and B) or nuclear extract (C) were subjected to SDS-PAGE, followed by immunoblotting and detection with Abs specific to STAT1. The trace is representative of three independent experiments.
RAW264.7 cells were cotransfected with the IRF-1-luciferase construct in the presence or the absence of IFN-α/H9253 and IFN-γ/H9253, and ~80% inhibition was achieved at 10 μM. Similar inhibitory extents by these PPARγ agonists on IFN-γ-induced IRF-1 activation were also seen in RAW264.7 macrophages (Fig. 5D).

To verify the PPARγ-independent action of 15dPGJ₂, we examined the effects of BADGE, a synthetic PPARγ antagonist. Fig. 6A shows that in the presence of BADGE (30 μM), PPARγ-driven PPRE trans-activation was abolished. Under this condition we found that ciglitazone- and GW1929-induced NO inhibition was reversed, while that of 15dPGJ₂ was still observed (Fig. 6B). Likewise, 15dPGJ₂-induced inhibition of STAT1 phosphorylation was not affected by BADGE (Fig. 6C). These results support our previous idea that 15dPGJ₂ can elicit a PPARγ-independent mechanism to inhibit JAK-STAT1 signaling.

15dPGJ₂ inhibition of IL-6-activated STAT3 phosphorylation

To clarify whether STAT1 inhibition by 15dPGJ₂ is unique to IFN-γ, we investigated its effect on IL-6-induced STAT3 and STAT1 signaling. In Hep3B hepatoma cells, IL-6 induced a time-dependent phosphorylation of STAT3 at Tyr701 (Fig. 7A). In the presence of 15dPGJ₂ (10 μM), STAT3 phosphorylation induced by IL-6 was reduced. A concentration-dependent effect of 15dPGJ₂ was detected within 1–30 μM (Fig. 7B). In addition to STAT3, IL-6 in Hep3B cells caused STAT1 phosphorylation at Tyr701, and this effect was reduced in parallel by 15dPGJ₂ (3–30 μM; Fig. 7C).

15dPGJ₂ inhibition abolished by antioxidants

Since several studies, including ours, have demonstrated the stimulating effect of 15dPGJ₂ on reactive oxygen species (ROS) production in macrophages (32), hepatic myofibroblasts (33), and C2C12 myocytes (34), we then examined the involvement of ROS production in STAT1 inhibition. In RAW264.7 macrophages, H₂O₂ (10 mM) and two antioxidants, N-acetylcysteine (NAC; 5 mM) and glutathione (GSH; 10 mM), themselves did not affect the basal level of STAT1 phosphorylation. In the presence of IFN-γ, H₂O₂ (1–10 mM) concentration-dependently reduced STAT1 phosphorylation and caused an additive inhibition with 10 μM 15dPGJ₂ (Fig. 8A). In the presence of either NAC or GSH, the STAT1 phosphorylation induced by IFN-γ was slightly, but significantly, enhanced. In the meanwhile, the inhibitory effect of 15dPGJ₂ on the IFN-γ response was abolished (Fig. 8B). In contrast, when cells were treated with superoxide dismutase and catalase to modulate intracellular ROS, 15dPGJ₂-elicited STAT1 phosphorylation was partially diminished (Fig. 8C). These results suggest that ROS production is an important key player in 15dPGJ₂ inhibition of JAK-STAT1 signaling.

Since JAKs play an intermediate and key role in transducing STAT1 signaling by IFN-γ, we next explored the effects of 15dPGJ₂ on JAK activation and the regulatory role of ROS in this signaling step. Since JAK2 autophosphorylation is regarded as an activated index, we performed an immunoblotting analysis. Fig. 8D indicates that IFN-γ-elicited JAK2 activation was reduced by 10 μM 15dPGJ₂ and 3 mM H₂O₂. In contrast, it was dramatically enhanced by ~3-fold in the presence of either NAC (5 mM) or assay, which was normalized by lacZ transfection efficiency, and expressed as a percentage of the control response without stimulus treatment. Results shown are the mean ± SEM from at least three independent experiments, each performed in duplicate. *p < 0.05; significant stimulation of PPARγ luciferase activity (B) or inhibition of IFN-γ-induced IRF-1 activity (C and D) by PPARγ agonists.
To explore the possibility that tyrosine phosphatase is a potential regulatory target for 15dPGJ$_2$, we examined the effect of peroxovanadate (POV), a protein tyrosine phosphatase inhibitor. As reported in peritoneal macrophages (35), POV alone did not induce NO release, but potentiated NO synthesis elicited by IFN-γ by 30%. Under this condition, 15dPGJ$_2$ still inhibited NO production, but to a smaller extent (Fig. 9A). Western blotting analysis showed that POV (30 μM) itself caused a weak increase in STAT1 phosphorylation in RAW264.7 macrophages, and this action was additive to that of IFN-γ. STAT1 phosphorylation induced by POV alone or in the presence of IFN-γ was reduced by 15dPGJ$_2$ (Fig. 9B). However, the absolute quantity of STAT1 phosphorylation under IFN-γ, POV, and 15dPGJ$_2$ treatment was higher than that under IFN-γ and 15dPGJ$_2$ treatment. Fig. 9C further shows the similar effects of POV in enhancing IL-6-induced STAT3 phosphorylation without changing the inhibitory action of 15dPGJ$_2$.

**Discussion**

IFN-γ is a key cytokine involved in the synergistic generation of many inflammatory responses. Studies in knockout mice have revealed that STAT1 is essential for enabling IFN-γ to synergize with bacterial LPS or TNF-α (9, 36). Given the opposite roles of IFN-γ in macrophage-related inflammatory responses and PPARγ in anti-inflammation, we studied the effects of PPARγ activators on IFN-γ-initiated iNOS induction in macrophages. In addition, the endogenous PPARγ activator, 15dPGJ$_2$, can be generated from foamy macrophages in human atherosclerotic plaques and LPS-stimulated RAW264.7 macrophages (37), and thus the anti-inflammatory effect of 15dPGJ$_2$ is pathologically implicated.

In line with the results observed by Ricote et al. (16) in peritoneal macrophages, where they examined 15dPGJ$_2$ and troglitazone, we demonstrate the ability of three PPARγ agonists to diminish NO production in response to IFN-γ in the murine macrophage RAW264.7 cell line. In this macrophage cell line, we detected the expression of PPARγ protein by immunoblotting analysis. Comparing the efficiency of PPARγ agonists for inhibition of IFN-γ-induced NO production, we observed a higher efficacy by 15dPGJ$_2$ than by ciglitazone or GW1929. At 10 μM, 15dPGJ$_2$ abolished NO production, while ciglitazone and GW1929 only reduced IFN-γ-induced NO production by 20–30%. The IC$_{50}$ of 15dPGJ$_2$ shown here also correlated with previous data (1 μM) concerning its inhibition of the LPS-induced iNOS/NO response.
Except for NO, 15dPGJ2 was also shown to be the most prominent PPAR/H9253 agonist for inhibiting cytokine (e.g., TNF-α, IL-1β, and IL-6) production (19) and cyclooxygenase-2 induction (21, 22). To explain this idea, previous studies identified several PPAR/H9253-independent targets of 15dPGJ2, such as IKK (19, 38), p65 NF-κB (39), p50 NF-κB (40), and Sp1 (41). These PPAR/H9253-independent actions provide additional anti-inflammatory mechanisms.

Based on nonspecific actions of 15dPGJ2, it is of great interest to further elucidate the action mechanism of 15dPGJ2 with respect to the IFN-γ response. First, we ruled out the possibility that the high sensitivity of 15dPGJ2 results from PPAR/H9253 activation based on two pieces of evidence. First, GW1929, the most potent PPAR/H9253 (10 mM), superoxide dismutase (SOD; 1000 U/ml), or catalase (10 mg/ml) for 30 min before the addition of vehicle, 15dPGJ2 (10 μM), and/or H2O2 (1–10 mM in A and 3 mM in D). Twenty minutes later, IFN-γ (10 ng/ml) was treated for 15 min. Tyrosine-phosphorylated STAT1 (A–C) and JAK2 (D) were measured by immunoblotting as previously described. Traces are representative of three independent experiments, and data for the histograms indicate the mean ± SEM from three independent experiments. *p < 0.05, significant inhibition of JAK/STAT signal pathway.

**FIGURE 8.** Inhibitory effect of 15dPGJ2 on STAT1 and JAK2 phosphorylation mimicked by H2O2, but prevented by antioxidants. As indicated, RAW264.7 cells were pretreated with vehicle, NAC (5 mM), GSH (10 mM), superoxide dismutase (SOD; 1000 U/ml), or catalase (10 mg/ml) for 30 min before the addition of vehicle, 15dPGJ2 (10 μM), and/or H2O2 (1–10 mM in A and 3 mM in D). Twenty minutes later, IFN-γ (10 ng/ml) was treated for 15 min. Tyrosine-phosphorylated STAT1 (A–C) and JAK2 (D) were measured by immunoblotting as previously described. Traces are representative of three independent experiments, and data for the histograms indicate the mean ± SEM from three independent experiments. *p < 0.05, significant inhibition of JAK/STAT signal pathway.

**FIGURE 9.** 15dPGJ2 action is independent of tyrosine phosphatase. RAW264.7 macrophages (A and B) and Hep3B cells (C) were pretreated with POV (30 μM) for 15 min, followed by vehicle or 10 μM 15dPGJ2 for 20 min, and then stimulated with IFN-γ (10 ng/ml) or IL-6 (10 ng/ml) for another 15 min (B and C) or 24 h (A). STAT1 (B) and STAT3 (C) immunoreactivities and nitrite production (A) after cytokine treatment were measured. Results in A are the mean ± SEM from at least three independent experiments, each performed in duplicate. *p < 0.05, significant inhibition upon 15dPGJ2 treatment. Results in B and C are representative of three independent experiments.
agonist, with an EC50 of 100 nM (30), could not efficiently reduce the IFN-γ response as well as 15dPGJ2. Second, the PPARγ antagonist BADGE (42), at concentrations that blocked PPARγ activity, did not affect the inhibitory effect of 15dPGJ2 on NO production and STAT1 phosphorylation. Moreover, we were cautious about the specificity of other prostanoids in this respect. In this context, we found that neither PGD2 (30 μM) nor PGE2 (1 μM) elicited an inhibitory effect similar to that of 15dPGJ2 (data not shown). Thus, we suggest that the higher potency and efficiency of 15dPGJ2 in attenuating NO production triggered by IFN-γ are mainly accomplished through PPAR-γ-independent mechanisms.

IFN-γ-induced iNOS expression has been ascribed to the pivotal signaling event of JAK-STAT1. The common processes sequentially required for STAT1-linked gene induction include tyrosine phosphorylation, nuclear translocation, binding to cognate binding sites in the promoter, and gene trans-activation. Results from immunoblotting, EMSA, and the reporter assay performed in this study suggest an inhibitory ability by 15dPGJ2 on JAK-STAT1 signaling cascades. Concentrations of 15dPGJ2 required to effectively interrupt STAT1 signaling were within 1–30 μM and were correlated to the inhibition of NO production. Furthermore, this STAT1 uncoupling effect of 15dPGJ2 is not specific to IFN-γ. We found that IL-6-mediated STAT3 and STAT1 signalings were also inhibited by 15dPGJ2. Although ciglitazone and GW1929 showed no such inhibition on STAT1 phosphorylation and DNA binding, both induced weak inhibition (15%) in the IRF-1 promoter assay. This inhibition, as previously demonstrated, is due to PPARγ-linked competition for the limited transcriptional coactivators (24).

Tyrosine phosphorylation of STAT1 is balanced by tyrosine phosphatase. The increased Tyr phosphorylation by POV indeed confirms the existence of this homeostatic balance. However, since POV-induced STAT1 phosphorylation is reduced by 15dPGJ2, and 15dPGJ2 still exerts inhibition of IFN-γ—plus POV-induced STAT1 phosphorylation, the possible action of 15dPGJ2 on tyrosine phosphatase activity seems exclusive. Our results indicate that the inhibitory action of 15dPGJ2 on the upstream JAK-STAT1 signaling pathway can be compromised by eliminating the tyrosine dephosphorylation process. Besides tyrosine phosphatase, another mechanism that needs to be considered is cAMP. Indeed, it has been reported that a cAMP increase can inhibit JAK1-STAT activation (43–48). We thus addressed this point by two approaches: measuring intracellular cAMP levels and using a PKA inhibitor. In the cAMP assay we did not detect significant changes after 15dPGJ2 treatment (data not shown). When cells were pretreated with a PKA inhibitor (KT5720), the inhibitory effects of 15dPGJ2 on NO production and STAT1 phosphorylation were not altered (data not shown). Thus, we can also exclude the involvement of cAMP in the action of 15dPGJ2.

ROS have been established as second messengers and can activate multiple intracellular signaling pathways. To date, although a few studies have implied a potential role of ROS in STAT1 and STAT3 activation (49–51), their role in the JAK-STAT pathway is still unclear. In the study reported here we found that antioxidants (NAC and GSH) can abrogate 15dPGJ2’s inhibition of both JAK2 and STAT1, while H2O2 mimics the effects of 15dPGJ2. These findings together with previous observations showing the ability of 15dPGJ2 to induce ROS production (32–34) strongly suggest that ROS are intermediate mediators for the action of 15dPGJ2. Moreover, in this study we unexpectedly found that both antioxidants (NAC and GSH) could increase IFN-γ-elicited JAK2 and STAT1 signaling, especially JAK2 activation. These results strengthen the negative regulatory role of the intracellular oxidative state in the JAK-STAT1 signal cascade. Regarding the contradictory results of ROS on STAT activation as presently observed in macrophages and previously shown in fibroblasts, A-431 carcinoma cells (49), and vascular smooth muscle cells (50), we currently can provide no explanation, except for cell type specificity.

Besides causing STAT1-dependent gene regulation, higher doses of IFN-γ were also demonstrated to activate NF-κB through tyrosine phosphorylation of IkBs in some cell types (35, 52) or to enhance NF-κB activation induced by TNF-α (53). Molecular mechanisms of IFN-γ-enhanced NF-κB activity point to the involvement of a dsRNA-activated protein kinase R (54) and an unidentified tyrosine kinase (35). To determine whether the NF-κB element contributes to the stimulatory action of IFN-γ in macrophages, we performed EMSA experiments to assay NF-κB’s activity and used an NF-κB inhibitor (PDTC) to determine the role of NF-κB. However, both experiments consistently excluded the ability of IFN-γ to activate NF-κB in RAW 264.7 macrophages. With regard to another transcription factor, AP-1, we explored whether PKC, extracellular signal-regulated kinase (ERK), or p38 MAPK might be involved in IFN-γ–induced iNOS induction. Actually, in myocytes and microvascular endothelial cells, IFN-γ–induced iNOS gene expression requires ERK activity (43). In the present study data using pharmacological inhibitors of PKC (Ro 31–8220, GF109203X), MEK (PD98059), and p38 MAPK (SB203580) ruled out the involvement of AP-1 in the action of IFN-γ. On the contrary, the inhibitory results of both selective (AG490) and nonselective (genistein) JAK inhibitors confirmed the crucial role of JAK/STAT signaling in IFN-γ–induced NO production in macrophages.

In conclusion, PPARγ agonists can inhibit iNOS gene transcription via PPAR-γ-dependent competition with transcription cofactors. In addition to this common action of PPARγ agonists, we have demonstrated a novel action mechanism underlying the inhibition of IFN-γ-induced NO production by 15dPGJ2. 15dPGJ2 via ROS production interrupts the JAK-STAT1 signaling pathway caused by IFN-γ, which leads to inhibition of STAT1-mediated iNOS expression. This PPARγ-independent action of 15dPGJ2 has shed new light on the action mechanism of this endogenous anti-inflammatory mediator.

References


