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Nanomolar and Micromolar Effects of 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 on Amnion-Derived WISH Epithelial Cells: Differential Roles of Peroxisome Proliferator-Activated Receptors γ and δ and Nuclear Factor κB

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ABSTRACT

15-Deoxy $\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), an activator of peroxisome proliferator-activated receptor (PPAR)-γ and -δ, is a prostanoid metabolite with anti-inflammatory actions. In intrauterine tissues, proinflammatory cytokines and prostaglandins have been identified as playing key roles in the maintenance of pregnancy and the onset of labor. We investigated and compared the early (<3 h) effects of 15d-PGJ_2 with rosiglitazone (PPAR-γ ligand) and 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid (GW501516) (PPAR-δ ligand) on interleukin (IL)-1β–induced prostaglandin and cytokine production by amnion-derived WISH cells. We show that 15d-PGJ_2 exerts differential effects depending on concentration. At low concentrations (<0.1 μM), 15d-PGJ_2 inhibited IL-1β–stimulated prostaglandin E_2 (PGE_2) but not cytokine (IL-6/IL-8) production or cyclooxygenase-2 (COX-2) expression. This effect was attenuated by a PPAR-γ inhibitor [2-chloro-5-nitro-*N*-phenyl-benzamide (GW9662)], by transfection with a dominant-negative PPAR construct, and was reproduced by the PPAR-γ ligand rosiglitazone. At higher concentrations (1–10 μM), 15d-PGJ_2 inhibited IL-1β–stimulated PGE_2 and cytokine production and COX-2 expression, and this effect was not blocked by GW9662. Rosiglitazone at high concentrations (1–10 μM) stimulated PGE_2 production in the absence or presence of the dominant-negative PPAR. The PPAR-δ ligand GW501516 also inhibited IL-1β–stimulated PGE_2 production but only at high concentrations (1 μM). IL-1β–induced nuclear factor-κB (NF-κB) DNA binding activity was significantly inhibited by 15d-PGJ_2 (10 μM) and GW501516 (1 μM) but increased with 10 μM rosiglitazone. We conclude that 1) at low concentrations, 15d-PGJ_2 acts through a PPAR-γ signaling pathway; b) at higher concentrations, its actions are mediated most likely through other pathways such as activation of PPAR-δ and/or inhibition of NF-κB; and 3) rosiglitazone exerts PPAR-independent effects at high concentrations (>1 μM).

Proinflammatory cytokines have been shown to play crucial roles in the maintenance of human pregnancy and the initiation of parturition (Romero et al., 1993; Mitchell et al., 1995). The presence of intrauterine infection has been shown to result in the local expression and secretion of proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor-α, IL-6, and IL-8 (Romero et al., 1993; Dudley et al., 1996; Keelan et al., 1999a), which act locally on intrauterine cells to induce the release of inflammatory mediators, extracellular matrix-remodeling enzymes (So et al., 1992; Draper et al., 1995), and prostaglandins (PGs) through altered expression of prostanoid biosynthetic enzymes including fatty acid cyclooxygenase-2 (COX-2) (Trautman et al., 1996; Hansen et al., 1999; Kniss, 1999; Rauk and Chiao, 2000).

Although most studies to date have focused on the produc-

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ABBREVIATIONS: IL, interleukin; 15d-PGJ_2, 15-deoxy $\Delta^{12,14}$-prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; rosiglitazone, 5-(4-[2-(*N*-methyl-*N*-(2-piridyl)amino)ethoxy]benzyl thiazolidine-2,4-dione maleic acid salt; COX-2, cyclooxygenase-2; NF-κB, nuclear factor κB; GW9662, 2-chloro-5-nitro-*N*-phenyl-benzamide; GW501516, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)-phenoxy-acetic acid; Bay 11–7085, (*E*)3-[(4-*t*-butylphenyl)sulfonyl]-2-propenenitrile; PG, prostaglandin; iNOS, inducible nitric-oxide synthase; ERK, extracellular signal-related kinase; HRPO, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyl transferase; DTT, dithiothreitol; ANOVA, analysis of variance; PPRE, peroxisome proliferator-activated receptor response element; PPAR D/N, peroxisome proliferator-activated receptor dominant-negative construct.

tion of uterotonic PGs such as PGE_2 and $PGF_{2\alpha}$, there is evidence of an abundance of PGD_2 in the intrauterine environment during labor (Mitchell et al., 1982; Berryman et al., 1987). PGD_2, synthesized from PGH_2 via the action of PGD synthases (Helliwell et al., 2004a), is readily converted nonenzymatically into PGJ_2 and its metabolites 9-deoxy-Δ^9,Δ^{12}-13,14-dihydroprostaglandin D_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (Fitzpatrick and Wynalda, 1983; Kikawa et al., 1984; Shibata et al., 2002). These metabolites have been postulated to regulate a number of different cellular processes, including cell proliferation (Chinery et al., 1999), differentiation (Forman et al., 1995; Kliewer et al., 1995), apoptosis (Kim et al., 1993; Hashimoto et al., 2002), and inflammation (Harris et al., 2002). 15d-PGJ_2 can induce apoptosis in several cell types (Bishop-Bailey and Hla, 1999; Rohn et al., 2001; Chen et al., 2002; Hashimoto et al., 2002; Rovin et al., 2002), including trophoblast (Schaiff et al., 2000), amnion-derived WISH cells (Keelan et al., 2001), and JEG3 choriocarcinoma cells (Keelan et al., 1999b). It is also reported to inhibit the expression of proinflammatory cytokines (Jiang et al., 1998; Ricote et al., 1998; Asada et al., 2004), inducible nitric-oxide synthase (iNOS) (Colville-Nash et al., 1998; Petrova et al., 1999), and COX-2 expression (Boyault et al., 2001; Tsubouchi et al., 2001; Mendez and LaPointe, 2003). However, its mechanism of action is controversial. Some have reported that 15d-PGJ_2 acts as an endogenous ligand for the peroxisome proliferator activated receptor (PPAR)-γ (Jiang et al., 1998; Ricote et al., 1998; Tsubouchi et al., 2001), whereas others have argued that its main effects are mediated through the inhibition of the transcription factor NF-κB (Rossi et al., 2000; Straus et al., 2000; Cernuda-Morollon et al., 2001) and modulation of the mitogen-activated protein kinase pathway (Hortelano et al., 2000; Rossi et al., 2000), such as inhibition of ERK phosphorylation (Relic et al., 2004).

In gestational tissues, PPAR-γ has been localized to the amnion, choriodecidual, and placental membranes (Marvin et al., 2000; Waite et al., 2000; Dunn-Albanese et al., 2004) and plays an important role in trophoblast differentiation and placental vascularization (Barak et al., 1999). NF-κB is a crucial transactivator of multiple proinflammatory and antiapoptotic genes (Lawrence et al., 2002). Recent studies have demonstrated that 15d-PGJ_2 inhibits the signaling steps leading to NF-κB activation by sequestering coactivators needed for transcription (Li et al., 2000), by inhibition of IκB-α kinase activity (Mercurio and Manning, 1999; Rossi et al., 2000), and through the formation of covalent bonds with cysteine residues of the DNA binding domain of NF-κB subunits (Rossi et al., 2000; Straus et al., 2000; Cernuda-Morollon et al., 2001).

Most of the studies to date have investigated the effect of 15d-PGJ_2 at micromolar concentrations. It is interesting that Emi et al. (2004) reported recently that 15d-PGJ_2 exhibits biphasic effects that are concentration-dependent. At 3 μM, it was shown to induce cell proliferation, but at 10 μM, it was an inducer of apoptosis. In gestational tissues, 15d-PGJ_2 ($>$10 μM) has been shown to inhibit extravillous cytotrophoblast invasion and differentiation (Schaiff et al., 2000; Tarrade et al., 2001; Pavan et al., 2003b), leading to trophoblast apoptosis (Schaiff et al., 2000). A recent study also showed that at a high concentration ($>$10 μM), 15d-PGJ_2 exhibited anti-inflammatory properties by reducing lipopolysaccharide-stimulated IL-6, IL-8, and tumor necrosis factor-α production by amnion, choriodecidual, and placental cells in vitro, possibly through the inhibition of NF-κB activity (Lappas et al., 2002).

The present study was conducted as part of an evaluation of the role of 15d-PGJ_2 in gestational tissues. We investigated the early effects ($<$3 h) of 15d-PGJ_2, rosiglitazone (a more potent and specific pharmacological PPAR-γ agonist), and GW501516 (a PPAR-δ agonist) on basal and IL-1β–induced PG and cytokine production in the WISH cell line (which has been used extensively in the past as an amnion epithelial cell model) (Pavan et al., 2003a) to clarify the effects and the mechanism(s) of action of 15d-PGJ_2 at low (0.001–0.1 μM) and high (0.1–10 μM) concentrations. Specific inhibitors were used to clarify the respective roles of PPARs and NF-κB as targets for 15d-PGJ_2–induced effects.

Materials and Methods

Reagents. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Ham's F-12/Dulbecco's modified Eagle's media were obtained from Irvine Scientific (Santa Ana, CA), and penicillin/streptomycin/glutamine, fetal calf serum, normal horse serum, trypsin-EDTA, and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide were purchased from Invitrogen NZ Limited (Auckland, New Zealand). Hybond-P nitrocellulose membranes were purchased from Amersham Biosciences Inc. (Auckland, New Zealand). Roche complete protease inhibitor tablets and human recombinant IL-1β were purchased from Roche Diagnostics (Auckland, New Zealand) and Immunex (Seattle, WA), respectively. The PPAR dominant-negative construct (pSG5hPPAR$\gamma\Delta$500) was a gift from Dr. Joel Berger (Department of Molecular Endocrinology, Merck Research Laboratories, Rahway, NJ). Anti-β-actin, COX-2, and NF-κB p50 and p65 antibodies were purchased from Abcam Limited (Cambridge, UK), BD Biosciences (San Jose, CA), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Goat anti-rabbit IgG-horseradish peroxidase (HRPO) antibody was purchased from Sigma-Aldrich. 15d-PGJ_2, rosiglitazone, and GW501516 were purchased from Cayman Chemical (Ann Arbor, MI). GW9662 and valinomycin were generous gifts from Dr. Tim Willson (GlaxoSmithKline, Uxbridge, Middlesex, UK) and Dr Mark McKeage (Department of Pharmacology, University of Auckland, Auckland, New Zealand), respectively.

Cell Culture. WISH cells (American Type Culture Collection, Manassas, VA) were maintained in Ham's F-12/Dulbecco's modified Eagle's culture media supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin/glutamine at 37°C in 95% air/5% CO_2. Cells were plated in 24-well plates and treated with various test agents in triplicate. A 3-h time point was chosen to pre-empt the apoptotic changes observed in morphology of WISH cells after treatment with 15d-PGJ_2 (10 μM) for 8 h (Keelan et al., 2001). At the end of each treatment, media were collected for PGE_2 and cytokine measurements, and cells were lysed with lysis buffer (2% SDS, 8% glycerol, and 62.5 mM Tris, pH 6.8, protease inhibitor solution) for Western blotting. Cellular protein concentrations were measured using Bio-Rad DC protein assay (Bio-Rad Laboratories Pty Ltd, Auckland, New Zealand) according to the manufacturer's instructions.

PGE_2 Radioimmunoassay. PGE_2 was measured by radioimmunoassay as described previously (Simpson et al., 1998), and production was expressed as the percentage of control (mean ± S.E.M.) of at least three experiments performed in triplicate over 3 h. Radioactivity was measured in a β-scintillation counter (Amersham Biosciences, Uppsala, Sweden). Curve-fitting (smoothed spline) and data extrapolation were performed using onboard software (Ultraterm; PerkinElmer Wallac, Turku, Finland).

Cytokine ELISAs. IL-6 and IL-8 were measured using DuoSet ELISA reagents (R&D Systems, Minneapolis, MN). The procedure was followed according to the manufacturer's instructions. A SpectraMAX-250 ELISA plate reader (Molecular Devices, Sunnyvale, CA) was used to read the sample absorbance at 490 nm. Curve-fitting, data extrapolation, and data analysis were performed using SoftMax Pro V Software (Molecular Devices).

Immunocytochemistry. Immunocytochemical staining was carried out to investigate changes in protein expression. Cells were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS) (145.4 mM NaCl, 12.0 mM Na_2HPO_4, and 3.9 mM KH_2PO_4). After fixation, cells were incubated with primary antisera diluted in PBS containing Triton X-100 and 5% normal horse serum and were allowed to incubate overnight at 4°C. Cells were then washed and incubated with the appropriate biotinylated secondary anti-rabbit antibody for 1 h at room temperature followed by incubation with streptavidin-biotinylated HRPO conjugate (Amersham Biosciences) for another 1 h. Cells were washed and stained with 3′3′-diaminobenzidine. Photomicrographs were taken using a Leitz DML microscope (Leica Microsystems, Deerfield, IL) equipped with a JVC TK-1281 video camera (JVC Company of America, Wayne, NJ).

Western Blotting. Proteins (10 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) at 220 V for 40 min and transferred onto Hybond-P nitrocellulose membranes at 100 mA for 1 h. The membranes were blocked with 5% skim-milk powder in PBS-Tween buffer and incubated with anti-COX-2 or anti-β-actin in the presence of 5% skim-milk powder for 2 h at room temperature. Membranes were washed and incubated with HRPO-conjugated secondary antibody for another 2 h at room temperature. The membranes were washed once more, and bands were detected by enhanced chemiluminescence (ECL Western Blotting Detection Reagent; Amersham Biosciences) according to manufacturer's instructions and quantified by densitometry using ImageQuaNT (Amersham Biosciences).

Transfection. For reporter-driven assays, WISH cells were seeded in six-well plates (100,000 cells/well) and transfected with the PPAR response element (PPRE)-driven luciferase reporter plasmid (pTK-PPREx3-luc) (Forman et al., 1995) and β-actin promoter-driven chloramphenicol acetyl transferase (CAT) constructs (pβactin-CAT) using FuGENE 6 (Roche Diagnostics) as described previously (Marvin et al., 2000). In brief, transfection mixes (0.5–1 μg of DNA/well) were transfected into WISH cells according to the manufacturer's instructions. After 24 h, media were exchanged with treatment media containing the specified concentrations of 15d-PGJ_2, rosiglitazone, and GW501516 for 3 h, and cell extracts were prepared using CAT-ELISA lysis buffer (Roche Diagnostics) supplemented with 5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride. CAT and luciferase activity were assayed by CAT-ELISA and Luciferase assay reagent (Promega, Madison, WI) using a Spectra Max 250 plate reader (Molecular Devices) and a Wallac Qy 1250 MicroBeta TriLux Jet (PerkinElmer Wallac, Turku, Finland)-injecting microplate counter, respectively. In the dominant-negative experiments, WISH cells were seeded in 24-well plates (50,000 cells/well) and transfected with 0.5 μg/well pSG5hPPAR$\gamma\Delta$500 (Berger et al., 2000) and/or 0.5 μg/well pTK-PPREx3-luc for 24 h, followed by treatment with 15d-PGJ_2 or rosiglitazone in the presence or absence of IL-1β for 3 h, and PGE_2 production was measured by radioimmunoassay.

NF-κB Activity. Cells were lysed with a hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.1% Triton X-100, protease inhibitor cocktail), and nuclear extraction was performed using an extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, and 25% (v/v) glycerol, protease inhibitor cocktail) at 4°C. NF-κB activity in nuclear lysates was measured using the colorimetric NF-κB p50/p65 transcription factor assay kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions.

Statistical Analysis. Data were analyzed using ANOVA with post hoc Dunnett's test. A p value <0.05 was considered significant compared with control values. Data from at least three experiments performed in triplicate were normalized to control and were expressed as a percentage of control mean ± S.E.M.

Results

We have reported previously that 15d-PGJ_2 (10 μM) induces apoptosis in amnion-derived WISH cells, which was detectable within 8 h of treatment (Keelan et al., 2001). The present study was conducted to investigate the early effects of 15d-PGJ_2 on WISH cells; hence, a 3-h time point was chosen to allow the study of signaling effects before the onset of apoptosis. No morphological evidence of apoptosis was observed within this time point (data not shown). To confirm that the cells were not in the early stages of apoptosis, the mitochondrial membrane potential of WISH cells was assessed using a dual-emission fluorescent dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, after treatment with 15d-PGJ_2 (10 μM) and valinomycin (1 μM), a K^+ ionophore that dissipates membrane potential, as a positive control. At 3 h, the membrane potential of 15d-PGJ_2–treated cells was not significantly different from that of untreated control cells. In contrast, valinomycin (1 μM) caused a significant reduction in mitochondrial membrane potential as early as 30 min, confirming that the 15d-PGJ_2–treated cells were not in the early stages of apoptosis (data not shown).

To assess the anti-inflammatory effect of 15d-PGJ_2, WISH cells were treated with 15d-PGJ_2 (0–10 μM) for 3 h, and media were collected for measurement of PGE_2 and cytokine (IL-6 and IL-8) production. Basal PGE_2, IL-6, and IL-8 production rates were 5.39, 0.09, and 0.08 pg/ml/mg protein/3 h, respectively. Treatment with 15d-PGJ_2 significantly inhibited basal PGE_2, IL-6, and IL-8 production but only at the highest concentration tested (Fig. 1). Assay interference precluded the measurement of PGE_2 production at 15d-PGJ_2 concentrations >1 μM. At 1 μM, 15d-PGJ_2 inhibited PGE_2 production to 70.69 ± 13.4% of vehicle control (mean ± S.E.M.). At 10 μM, 15d-PGJ_2 significantly inhibited IL-6 and IL-8 production to 55.25 ± 12.1% and 23.35 ± 13.4% of control, respectively (Fig. 1).

The inhibitory effect of 15d-PGJ_2 was much more pronounced on cells stimulated with IL-1β. 15d-PGJ_2 significantly inhibited IL-1β–induced PGE_2 production by 50 to 60%, even at the lowest concentration tested (0.001 μM) (Fig. 2A). It is interesting that the PPAR-γ–specific inhibitor GW9662 (10 μM) attenuated the inhibitory effect of 15d-PGJ_2 at the lower concentrations of 15d-PGJ_2 tested (0–0.1 μM) but not at the highest concentration (1 μM) (Fig. 2A). 15d-PGJ_2–mediated reduction of cytokine production was also more pronounced in the presence of IL-1β (Fig. 2, B and C). At 30 μM, IL-1β–stimulated IL-6 and IL-8 production were significantly inhibited by 15d-PGJ_2 to 13.73 ± 6.8% and 14.97 ± 4.6% of control, respectively. The high-dose effects of 15d-PGJ_2 on IL-6 or IL-8 production were not significantly attenuated by GW9662, suggesting that 15d-PGJ_2 exerts both PPAR-γ–dependent (low concentration) and -independent (high concentration) effects on IL-1β–induced WISH cells.

In light of the importance of COX-2 transcription in cytokine-stimulated prostaglandin production, the effect of 15d-PGJ_2 on the amounts of IL-1β–induced COX-2 protein was also investigated. COX-2 protein is an inducible enzyme that was undetectable by immunoblotting in WISH cells under basal conditions. IL-1β–induced COX-2 protein expression was significantly inhibited by treatment with 15d-PGJ_2 at concentrations of 0.1 to 10 μM, whereas no changes were observed in response to GW9662 (10 μM) (Fig. 3). GW9662 on its own had no significant effects on COX-2 protein amounts (data not shown).

These data support the conclusion that at higher concentrations, 15d-PGJ_2 may be acting through a PPAR-γ–independent pathway, whereas at low doses, PPAR-γ activation may be involved. To test this hypothesis we examined the effects of the pharmacological PPAR-γ ligand rosiglitazone on prostaglandin and cytokine production. Under basal conditions, rosiglitazone had no effect on PGE_2 production except at high concentrations (10 μM) where, paradoxically, it significantly increased basal PGE_2 production to 155.95 $\pm$ 17.75% of control (Fig. 4A). In IL-1β–stimulated cells, rosiglitazone inhibited PGE_2 production at low concentrations (0.001–0.01 μM), and this inhibitory effect was significantly abolished by PPAR-γ blockade with GW9662 (Fig. 4B). At higher concentrations (>0.1 μM), rosiglitazone again induced a concentration-dependent increase in IL-1β–induced PGE_2 production up to 632.8 $\pm$ 150.7% of control at 10 μM. GW9662 only partially diminished rosiglitazone-induced stimulation of PGE_2 production (Fig. 4B). Rosiglitazone had no significant effect on COX-2 expression or IL-6 or IL-8 production (data not shown).

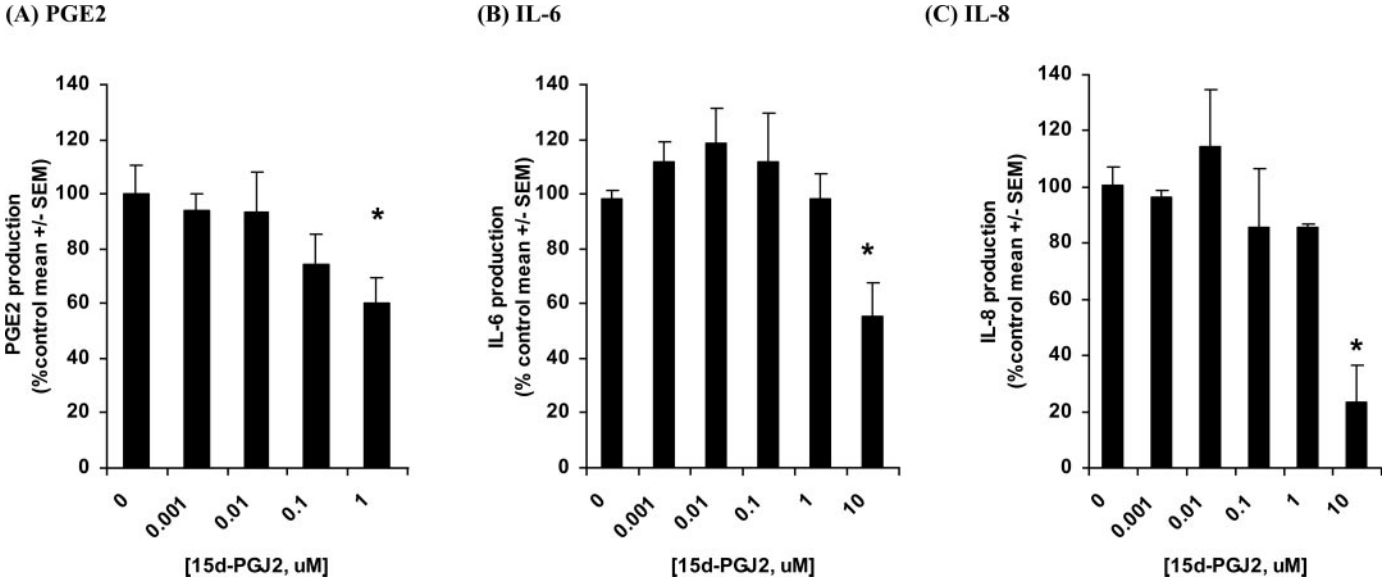

Fig. 1. The effect of 15d-PGJ_2 (0–10 μM) on PGE_2 (A), IL-6 (B), and IL-8 (C) production after 3 h. Basal rates of production of PGE_2, IL-6, and IL-8 were 5.39, 0.09, and 0.08 pg/ml/mg protein/3 h, respectively. Results are expressed as a percentage of control (mean $\pm$ S.E.M., n = 3–6 independent experiments performed in triplicate). $\star$, $p < 0.05$ by ANOVA with post hoc Dunnett's test.

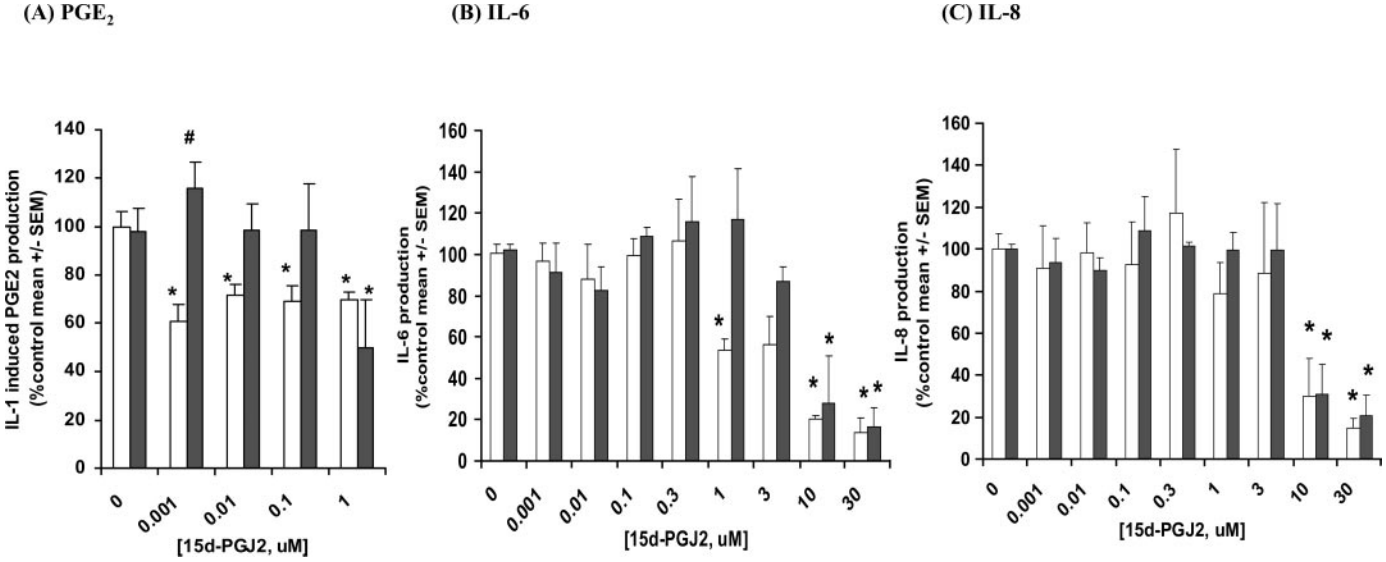

Fig. 2. The effect of 15d-PGJ_2 (0–10 μM) on IL-1β–induced PGE_2 (A), IL-6 (B), and IL-8 (C) production in the absence (□) or presence (▨) of GW9662 (10 μM) after 3 h. Results are expressed as a percentage of control (mean $\pm$ S.E.M., n = 3–6 independent experiments performed in triplicate). $\star$, $p <$ 0.05 by ANOVA with post hoc Dunnett's test; #, significant difference in PGE_2 production in IL-1β versus IL-1β plus GW9662 treatment.

15d-PGJ$_2$ has been reported to be an activator of PPAR-δ and -γ (Forman et al., 1995, 1997; Kliewer et al., 1995; Ferry et al., 2001). To assess the relative roles of PPAR-γ and -δ in mediating 15d-PGJ$_2$ effects, WISH cells were transfected with a pTK-PPREx3-luc reporter plasmid, and luciferase activity was determined after treatment with 15d-PGJ$_2$ (0–10 μM), the PPAR-γ ligand rosiglitazone (0–10 μM), and the PPAR-δ ligand GW501516 (0–1 μM). At the highest concentration tested, 15d-PGJ$_2$ (10 μM) and GW501516 (1 μM) significantly increased luciferase activity to 1.84- ± 0.6-fold and 1.49 ± 0.1-fold, respectively (Fig. 5). In contrast, rosiglitazone stimulated luciferase activity at a lower concentration (0.1 μM), causing a 1.6 ± 0.4-fold increase in luciferase activity observed (Fig. 5).

To further clarify the involvement of PPAR activation in the high and low concentration effects of 15d-PGJ$_2$ and rosiglitazone, WISH cells were transfected with a PPAR dominant-negative construct (PPAR D/N), pSG5hPPARγΔ500, a deletion mutant that lacks five amino acid at its carboxyl terminus (Berger et al., 2000). We first assessed the activity of this construct by cotransfecting pSG5hPPARγΔ500 with a pTK-PPREx3-luc reporter plasmid and assessing the level of PPAR-driven luciferase activity after stimulation with rosiglitazone (10 μM). Basal and rosiglitazone-induced luciferase activity was significantly suppressed in the presence of pSG5hPPARγΔ500 (Fig. 6A). Next, PGE$_2$ production of transfected cells was measured after treatment with 15d-PGJ$_2$ (0–10 μM) and rosiglitazone (0–10 μM). Transfection

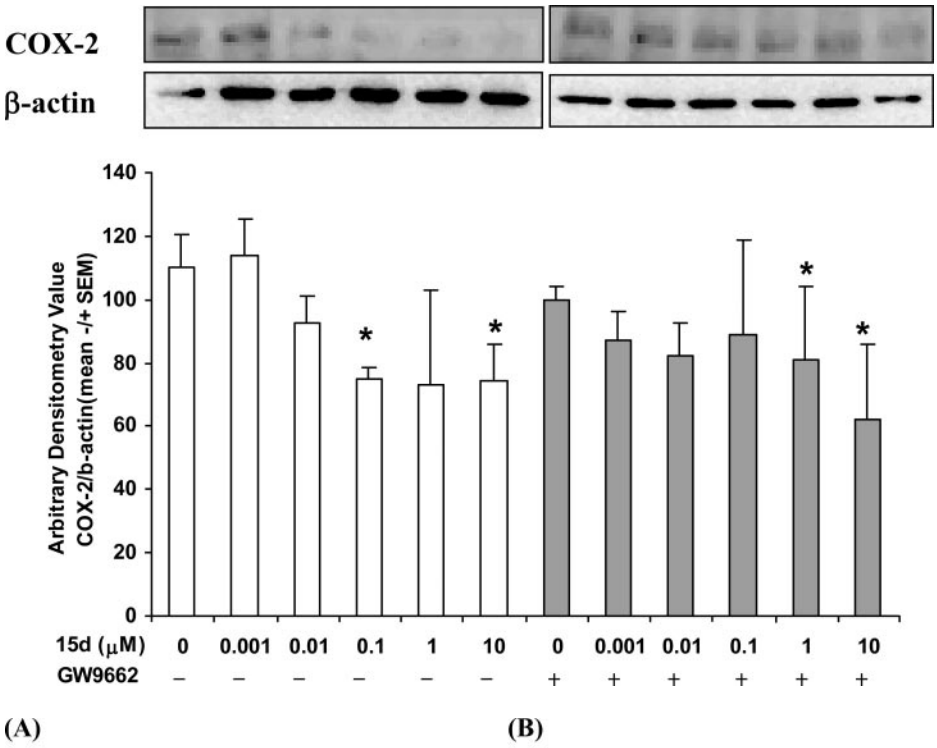

Fig. 3. The effect of 15d-PGJ$_2$ (0–10 μM) on IL-1β–induced COX-2 protein in the absence (□) and presence (▨) of GW9662 (10 μM) was measured by Western blotting. Blot is a representative of three separate experiments. Mean densitometry values of COX-2 normalized to β-actin are presented as a percentage of control (mean ± S.E.M.). ⋆, $p < 0.05$ by ANOVA with post hoc Dunnett's test.

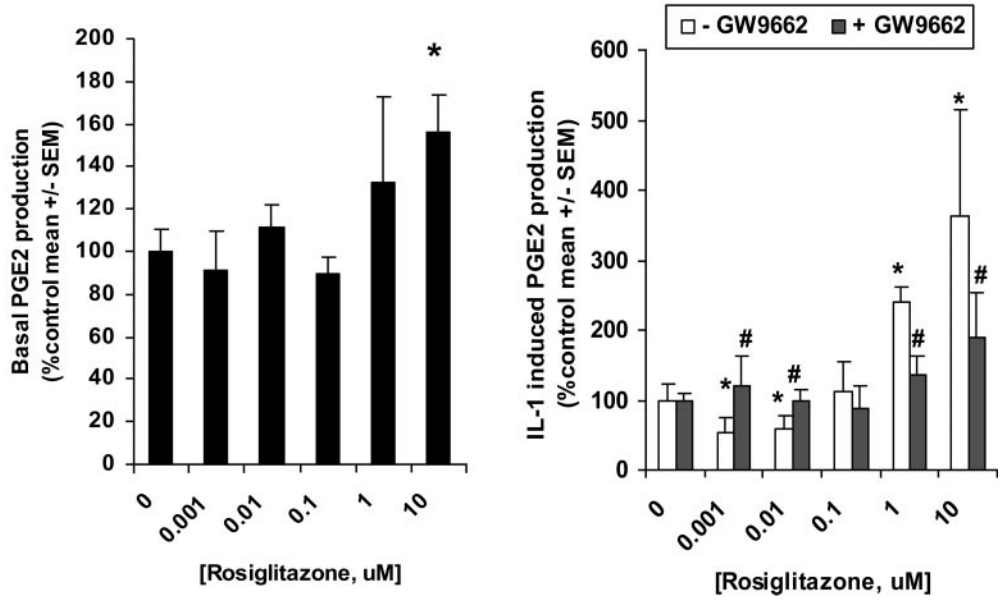

Fig. 4. The effect of rosiglitazone (0–10 μM) on basal PGE$_2$ (A) and IL-1β (B)–induced PGE$_2$ production in the absence (□) or presence (▨) of GW9662 (10 μM) after 3 h of treatment. Results are expressed as a percentage of control (mean ± S.E.M., n = 3–6 independent experiments performed in triplicate). ⋆, $p < 0.05$ by ANOVA with post hoc Dunnett's test; #, PGE$_2$ production in IL-1β versus IL-1β plus GW9662 treatment.

with PPAR D/N increased basal PGE_2 production by ~20-fold; it also abolished the high- and low-dose effect of 15d-PGJ_2 on PGE_2 production (Fig. 6B). The construct also suppressed the effects of rosiglitazone on PGE_2 production except at high concentrations (10 μM), at which a significant increase in PGE_2 production (397.2 $\pm$ 75.2% of control) remained (Fig. 6C). These data suggest that the inhibitory effects of 15d-PGJ_2 and rosiglitazone are PPAR-dependent, whereas the stimulatory high-dose effect of rosiglitazone is PPAR-γ–independent.

To determine whether PPAR-δ activation could initiate some of the responses observed with 15d-PGJ_2 treatment, WISH cells were treated with GW501516 (0–1 μM) in the absence and presence of IL-1β (0.2 ng/ml), and media were collected for PGE_2 measurements. PPAR-δ agonism with GW501516 had no significant effect on basal PGE_2 production (data not shown), but at 1 μM, it significantly inhibited IL-1β–induced PGE_2 production to 47.55 $\pm$ 6.3% of control (Fig. 7). Together, we interpreted these findings as indicating that PPAR-δ activation might contribute to the high-dose inhibitory effect of 15d-PGJ_2, but not at the low dose.

We next investigated the effect of 15d-PGJ_2 on NF-κB activation, this being the most likely alternative mechanism through which 15d-PGJ_2 might be exerting its effects. We performed immunocytochemical studies to examine the effect of 15d-PGJ_2 on nuclear translocation of NF-κB p65 subunit.

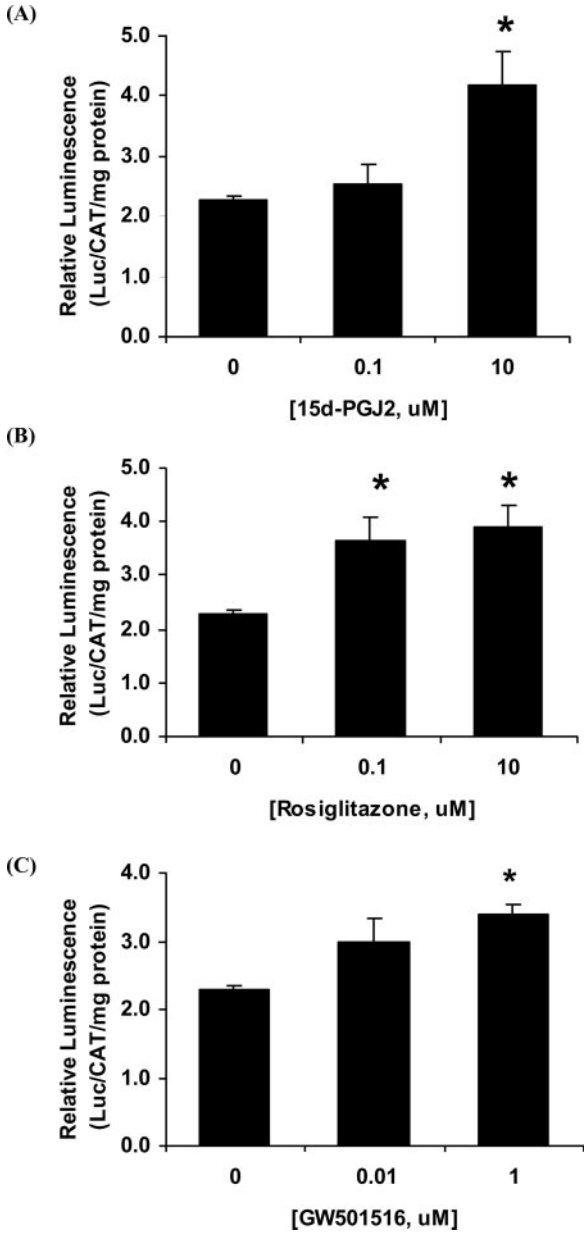

Fig. 5. The effect of PPAR-γ ligands 15d-PGJ_2 (A), rosiglitazone (B), and PPAR-δ ligand GW501516 (C) on the induction of pTK-PPREx3-luc luciferase reporter activity. The data represent three experiments performed in triplicate. $\star$, $p < 0.05$ was considered significant by ANOVA with post hoc Dunnett's test.

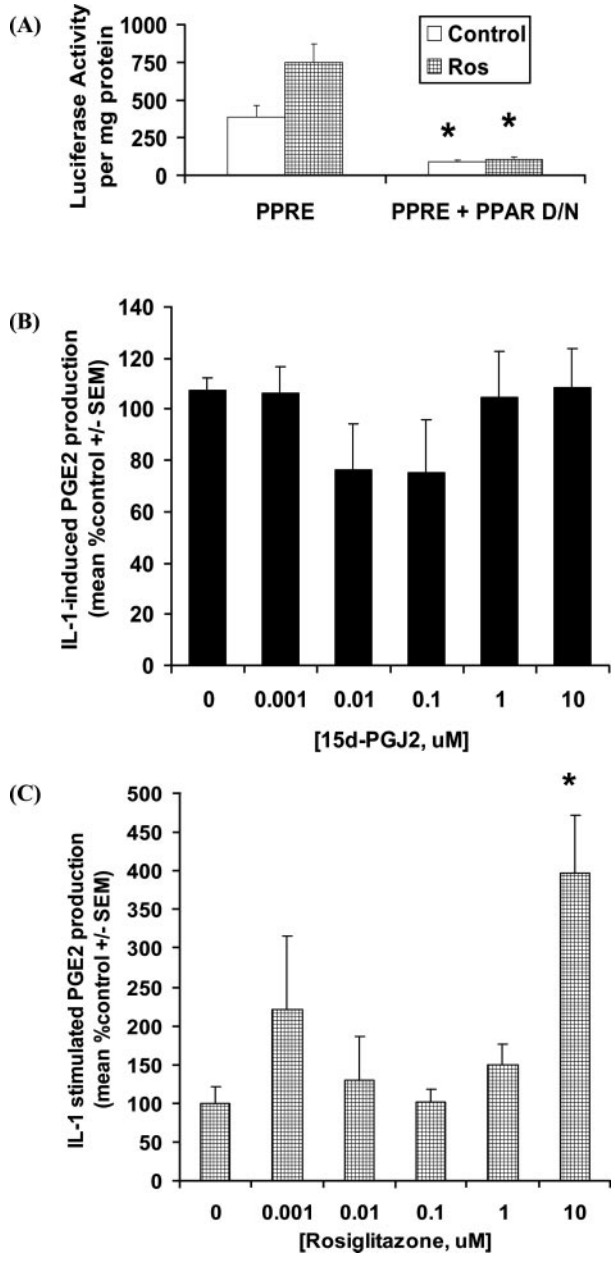

Fig. 6. A, the effect of pSG5hPPAR$\gamma\Delta$500 (a PPAR D/N construct) on basal and rosiglitazone-induced luciferase activity in cells cotransfected with pSG5hPPAR$\gamma\Delta$500 and pTK-PPREx3-luc. B, PGE_2 production in pSG5hPPAR$\gamma\Delta$500-transfected cells in response to treatment with IL-1β and 15d-PGJ_2 (0–10 μM). C, PGE_2 production in pSG5hPPAR$\gamma\Delta$500-transfected cells after stimulation with IL-1β and rosiglitazone (0–10 μM). $\star$, $p < 0.05$ by ANOVA with post hoc Dunnett's test.

Cytoplasmic localization was observed in untreated cells. Treatment with IL-1β led to a modest increase in nuclear p65 immunostaining. 15d-PGJ$_2$ (10 μM) did not markedly inhibit IL-1β–stimulated nuclear localization of p65 after 3 h of treatment (Fig. 8A), although the extent of nuclear staining seemed somewhat diminished. To further explore the effects of 15d-PGJ$_2$ on the NF-κB pathway, the effect of low/high doses of 15d-PGJ$_2$ (0.1 and 10 μM) on IL-1β–induced NF-κB activity was investigated using a DNA binding-immunoassay technique together with rosiglitazone (10 μM), GW501516 (1 μM), and the NF-κB inhibitor Bay 11-7085 (40 μM) as a control (Fig. 8B). IL-1β treatment induced a 66.17 $\pm$ 19% increase in nuclear NF-κB activity which was inhibited by Bay 11-7085. NF-κB activity was also inhibited to a lesser extent by 15d-PGJ$_2$ and GW501516. However, 10 μM rosiglitazone induced a 30% increase in IL-1β–induced NF-κB activity, consistent with its ability to stimulate PGE$_2$ production at this dose (Fig. 8B).

Discussion

In pregnancy, inflammatory processes have been shown to play roles in the mechanisms of preterm labor and preterm premature rupture of membranes as well as in normal term labor. Significant progress has been made in defining the nature of the immunological response that occurs within gestational membranes in the face of inflammatory activation and the cascade of events that leads to the production and metabolism of prostanoids and other lipid-derived mediators (Bowen et al., 2002; Keelan et al., 2003).15d-PGJ$_2$, a PGD$_2$ metabolite, has been studied extensively after its elucidation as a PPAR-γ ligand. It has been shown to inhibit the expression of a variety of proteins with proinflammatory properties, including COX-2 (Boyault et al., 2001; Tsubouchi et al., 2001; Mendez and LaPointe, 2003), iNOS (Colville-Nash et al., 1998; Petrova et al., 1999), and cytokines (Daynes and Jones, 2002), both in vitro and in animal models of autoimmune and inflammatory disease (Kawahito et al., 2000; Reilly et al., 2000; Diab et al., 2002). The intracellular accumulation of 15d-PGJ$_2$ in vivo has been demonstrated (Shibata et al., 2002), and 15d-PGJ$_2$ concentrations have been measured recently in biological fluids at picomolar amounts (Bell-Parikh et al., 2003). However, most of the findings to date have investigated the effects of 15d-PGJ$_2$ at micromolar concentrations that greatly exceed those associated with the biologic activity of conventional prostaglandins (picomolar to nanomolar concentrations) (Mitchell et al., 1978a,b). We are the first to report the effects of 15d-PGJ$_2$ at concentrations as low as 1 nM, effects that are evident even as early as 3 h. The finding of low-dose effects supports the notion that 15d-PGJ$_2$ may be a mediator of real physiological significance.

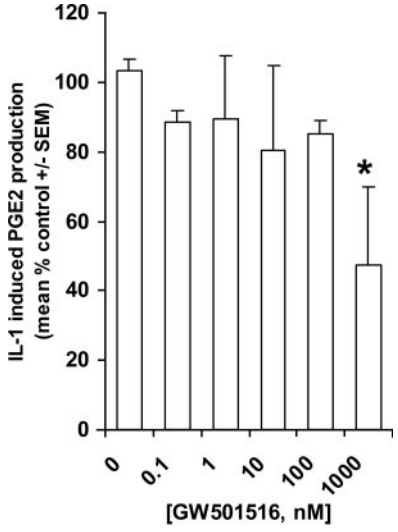

Fig. 7. The effects of PPAR-δ ligand GW501516 (0–1000 nM) on IL-1β–induced PGE$_2$ production after 3 h of treatment (n = 3 experiments performed in triplicate). $\star$, $p < 0.05$ by ANOVA with post hoc Dunnett's test.

The actions of 15d-PGJ$_2$ seem to be mediated through multiple mechanisms, depending partly on its concentration. 15d-PGJ$_2$ inhibited production of both basal and IL-1β–induced PGE$_2$, IL-6, and IL-8, with a greater level of inhibition observed in IL-1β–stimulated conditions. Our findings support the interpretation that 15d-PGJ$_2$, at low concentrations ($<$0.1 μM), exerts its anti-inflammatory effects through the activation of PPAR-γ because the effects were mimicked by rosiglitazone, were partially reversed by GW9662, and were absent in the presence of a dominant-negative PPAR construct. It is interesting that at high concentrations ($\sim$100 times its EC$_{50}$ for PPAR-γ activation), rosiglitazone induced a paradoxical increase in IL-1β–stimulated PGE$_2$ production that was only partially inhibited in the presence of GW9662. This stimulatory effect remained apparent in the presence of the dominant-negative construct, which suggests that the response to high concentrations of rosiglitazone is PPAR-independent. Increased ERK1/2 phosphorylation (Ruiz et al., 2004) and mitogen-activated protein kinase phosphorylation (Camp and Tafuri, 1997; Chen et al., 2003) have been documented in other cell types in response to rosiglitazone and would be potential explanations for this phenomenon.

Although our results indicate PPAR-γ as a likely candidate, we cannot discount the involvement of other PPAR isoforms in the effects observed because 15d-PGJ$_2$ has been reported to have similar affinities for both PPAR-γ and -δ (Forman et al., 1995, 1997; Helliwell et al., 2004b). In the present study, we found that the PPAR-δ ligand GW501516 (1 μM) was also active in inhibiting IL-1β–induced PGE$_2$ production, suggesting that the high concentration effect of 15d-PGJ$_2$ in WISH cells may be mediated, at least in part, through the activation of PPAR-δ. We have recently published data that support this argument, showing that the PPAR-γ antagonist GW9662 is only partially effective at inhibiting 15d-PGJ$_2$–induced activation of PPRE-driven reporter in JEG3 cells, whereas it completely abolished the rosiglitazone effect (Berry et al., 2003). The inhibition of 15d-PGJ$_2$ effects observed with the PPAR dominant-negative construct further supports the conclusion that 15d-PGJ$_2$ mediates its anti-inflammatory activity through the activation of PPARs because the PPAR D/N construct inhibits the transcriptional activity of all three PPAR isoforms (Berger et al., 2000). These data do not allow us to conclude whether blockade of PPAR-γ or PPAR-δ is responsible for abolishing either the low- or high-dose effect of 15d-PGJ$_2$ (WISH cells do not express PPAR-α) (Berry et al., 2003). Further studies are required to confirm the specific roles of the two PPAR isoforms in the inhibition of PGE$_2$ by 15d-PGJ$_2$ and rosiglitazone.

Inhibition of NF-κB activity is a well-documented anti-in-

flammatory pharmacotherapeutic approach, and the NF-κB pathway has been demonstrated to be a major target of 15d-PGJ_2 (Jiang et al., 1998; Rossi et al., 2000; Straus et al., 2000; Cernuda-Morollon et al., 2001). In our studies, high concentrations (10 μM) of 15d-PGJ_2 inhibited DNA binding by NF-κB after 3 h of treatment. Similar findings have been reported in several other studies in other tissues (Rossi et al., 2000; Straus et al., 2000; Boyault et al., 2001; Cernuda-Morollon et al., 2001). The failure of rosiglitazone to reproduce the effect supports the conclusion that 15d-PGJ_2 acts on NF-κB through a mechanism that is independent of PPAR-γ. This is consistent with a recent work by Lappas et al. (2002), which showed that 15d-PGJ_2 (30 μM) but not troglitazone (a PPAR-γ agonist) inhibited lipopolysaccharide-induced cytokine production through suppression of NF-κB DNA binding activity in gestational tissues (Lappas et al., 2002). However, it is noteworthy that the concentration of 15d-PGJ_2 used in that study was 3 to 30 times higher than that used in the present study.

Both PPAR activation and NF-κB inhibition require changes in gene transcription to effect an anti-inflammatory response. With respect to its effects on PGE_2 production, we anticipated that 15d-PGJ_2 would act via inhibition of COX-2

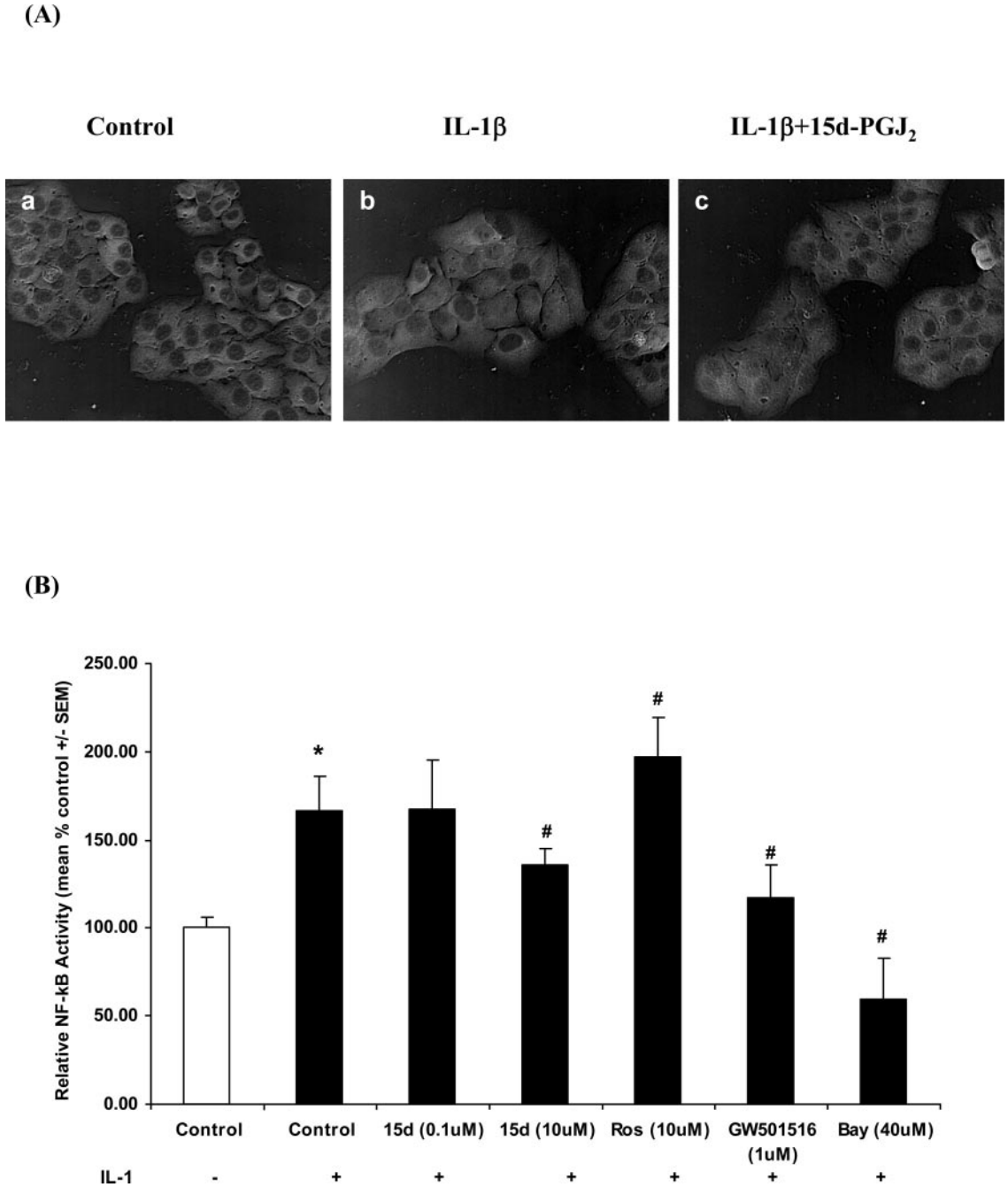

Fig. 8. A, immunocytochemical analysis of NF-κB p65 subunit localization in WISH cells treated with vehicle control (a), IL-1β (0.2 ng/ml) (b), and IL-1β and 15d-PGJ_2 (10 μM) (c) for 3 h. Photomicrographs are displayed as negative images for visual clarity (magnification, 40×). The data presented are representative of at least three independent experiments. B, effect of 15d-PGJ_2, rosiglitazone, GW501516, and Bay 11–7085 (Bay) on IL-1–stimulated (■) NF-κB DNA binding activity using an ELISA-based technique ($n = 3$ experiments performed in duplicate). ⋆, $p < 0.05$ compared with basal (control) by ANOVA with post hoc Dunnett's test. #, $p < 0.05$ compared with IL-1β–stimulated.

expression through PPAR-dependent or NF-κB–dependent mechanisms, as has been shown previously in other tissues (Inoue et al., 2000; Sawano et al., 2002; Mendez and LaPointe, 2003). However, the reduction in PGE_2 production in WISH cells by nanomolar concentrations of 15d-PGJ_2 occurred independently of COX-2 protein levels. Alternative mechanisms might be inhibition at the level of either COX-2 activity or arachidonate release by phospholipases, both of which would also be consistent with the relatively rapid changes in PGE_2 production reported here. COX-2 activity may be inhibited through 15d-PGJ_2's ability to deplete intracellular glutathione levels because apocynin, a compound that depletes intracellular glutathione through the inhibition of NADPH oxidase activity, was able to inhibit COX-2 production and this was reversed in the presence of a GSH precursor (Barbieri et al., 2004). 15d-PGJ_2 can also directly modify cellular thiol-containing proteins to reduce the activity of enzymes such as iNOS (Sanchez-Gomez et al., 2004) and microsomal prostaglandin E synthase (Murakami et al., 2000), the latter being the enzyme that catalyzes the biosynthesis of PGE_2. Finally, we cannot rule out the possibility that other targets and mechanisms might be involved. For example, Ruiz et al. (2004) recently reported that 15d-PGJ_2 (>10 μM) inhibited lipopolysaccharide-stimulated IL-6 gene expression in CMT-93 cells through the activation of protein phosphatase 2A activity and induction of ERK phosphorylation. Further studies are required to address these alternative possibilities.

In conclusion, we report that 15d-PGJ_2 exerts its anti-inflammatory effects in WISH cells through several pathways depending on its concentration. At low concentrations (≤0.1 μM), the effect of 15d-PGJ_2 seem to be mediated through the activation of PPAR-γ; however, at higher concentrations (>0.1 μM), activation of PPAR-δ and/or inhibition of NF-κB are involved. The abundance of PGD_2 in the amniotic cavity allows for the possibility that its metabolite 15d-PGJ_2 might exert anti-inflammatory actions in the uterus via one, or both, of these mechanisms. To what extent such effects are significant in the context of the inflammatory reaction that occurs in term and preterm labor remains to be determined.

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