

Anti-Inflammatory Actions of 15-Deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ and Troglitazone

Evidence for Heat Shock-Dependent and -Independent Inhibition of Cytokine-Induced Inducible Nitric Oxide Synthase Expression

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In this study, the anti-inflammatory actions of the peroxisome proliferator-activated receptor (PPAR)- γ agonists 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15-d- $\Delta^{12,14}$ -PGJ₂) and troglitazone have been examined. Treatment of RAW 264.7 cells and CD-1 mouse peritoneal macrophages with lipopolysaccharide (LPS) + interferon- γ (IFN- γ) results in inducible nitric oxide synthase (iNOS), inducible cyclooxygenase (COX-2) and interleukin-1 (IL-1) expression, increased production of nitric oxide, and the release of IL-1. In a concentration-dependent manner, 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits each of these proinflammatory actions of LPS + IFN- γ , with half-maximal inhibition at ~0.5 μ g/ml and complete inhibition at 1–5 μ g/ml. The inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced inflammatory events are not associated with the inhibition of iNOS enzymatic activity or macrophage cell death, but appear to result from an inhibition of iNOS and IL-1 transcription. In addition, the anti-inflammatory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ are not limited to peritoneal macrophages, as 15-d- $\Delta^{12,14}$ -PGJ₂ prevents TNF- α + LPS-induced resident islet macrophage expression of IL-1 β and β -cell expression of iNOS stimulated by the local release of IL-1 in rat islets. 15-d- $\Delta^{12,14}$ -PGJ₂ appears to be ~10-fold more effective at inhibiting resident islet macrophage activation (in response to TNF + LPS) than IL-1-induced nitrite production by β -cells. Two mechanisms appear to be associated with the anti-inflammatory actions of both 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone: 1) the direct inhibition of cytokine- and

endotoxin-stimulated iNOS and IL-1 transcription; and 2) the inhibition of IL-1 signaling, an event associated with PPAR- γ agonist-induced activation of the heat shock response (as assayed by heat shock protein 70 expression). These findings indicate that the PPAR- γ agonists, troglitazone and the J series of prostaglandins, are potent anti-inflammatory agents that prevent cytokine- and endotoxin-stimulated activation of peripheral and resident tissue macrophages and cytokine-induced iNOS expression by β -cells by the inhibition of transcriptional activation and induction of the heat shock response. *Diabetes* 49:346–355, 2000

Autoimmune diabetes is characterized by a local inflammatory reaction in and around the islets of Langerhans that is followed by selective destruction of β -cells (1). Cellular components of islet inflammation include T-cells, monocytes, and macrophages (2,3). Studies performed in both the nonobese diabetic (NOD) mouse and the Biobreeding (BB) rat suggest that macrophages play a primary role in the development of autoimmune diabetes. In the NOD mouse, macrophages appear to comprise the primary infiltrates into islets at early stages of insulinitis (3,4). In both the BB rat and NOD mouse, macrophage inactivation by silica treatment prevents the development of insulinitis and diabetes (5,6). In addition, macrophage depletion by treatment with liposome-encapsulated dichloromethylene diphosphonate prevents diabetes induced by Kilham rat virus in BB rats (7).

It has been suggested that macrophages may mediate the initial destruction of β -cells during the development of autoimmune diabetes (8). Administration of a diet deficient in essential fatty acids prevents the natural development of diabetes in BB rats (9) and multiple low-dose streptozotocin-induced diabetes in CD-1 mice (10). This fatty acid-deficient diet appears to prevent the development of diabetes by depleting resident tissue macrophages (8,11). In addition, Lacy and Finke (12) have shown that depletion of class II⁺ cells from isolated islets prevents interferon- γ (IFN- γ)-induced islet degeneration. We have shown that treatment of isolated rat islets with tumor necrosis factor (TNF) + lipopolysaccharide (LPS),

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15-d- $\Delta^{12,14}$ -PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; AG, aminoguanidine; COX-2, inducible cyclooxygenase; hsp, heat shock protein; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, C-Jun NH₂-terminal kinase; LPS, lipopolysaccharide; MTT, 3-[4-(5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]; NF, nuclear factor; PCR, polymerase chain reaction; PEC, peritoneal exudate cell; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor.

or human islets with TNF + LPS + IFN- γ , results in an inhibition of insulin secretion that is mediated by resident macrophage expression and release of interleukin (IL)-1 β in islets followed by IL-1 β -induced inducible nitric oxide synthase (iNOS) expression by β -cells (13–15). Taken together, this evidence suggests that both resident islet and inflammatory macrophages participate in β -cell damage during the development of autoimmune diabetes.

Recently, peroxisome proliferator-activated receptors (PPARs) have been shown to display anti-inflammatory activity. The PPAR- α agonist leukotriene B4 has been shown to have anti-inflammatory activity, and in mice with homozygous knockout of PPAR- α , inflammation is prolonged (16). PPAR- γ expression appears to be upregulated in activated macrophages, and agonists of this receptor prevent cytokine-stimulated expression of iNOS, gelatinase B, and scavenger receptor A (17,18). In addition, PPAR- γ agonists have been shown to stimulate the heat shock response, as evidenced by the expression of heat shock protein (hsp) 70, heat shock factor-1, and heme oxygenase (19–21). We have shown that IL-1 fails to stimulate nuclear factor κ B (NF- κ B) nuclear translocation and inhibitory protein κ B (I κ B) degradation if islets are heat shocked before cytokine stimulation (22). In this report, we have further characterized the anti-inflammatory actions of the PPAR- γ agonist 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15-d- $\Delta^{12,14}$ -PGJ₂) and troglitazone on macrophage activation and β -cell production of nitric oxide. We show that the anti-inflammatory activities of these PPAR- γ agonists are associated: 1) with a direct inhibition of cytokine and endotoxin-induced transcriptional activation; and 2) with the inhibition of cytokine signaling, an event associated with induction of the heat shock response.

RESEARCH DESIGN AND METHODS

Materials and animals. Mouse macrophage RAW 264.7 cells and rat RINm5F insulinoma cells were obtained from Washington University Tissue Culture Support Center. RPMI 1640 containing 1 \times L-glutamine, CMRL-1066 tissue culture medium, L-glutamine, penicillin, streptomycin, and mouse and rat recombinant IFN- γ was from Gibco BRL (Grand Island, NY). Fetal calf serum was obtained from Hyclone (Logan, UT). Male CD-1 mice (20–24 g) and male Sprague Dawley rats (250–300 g) were purchased from Harlan (Indianapolis, IN). All prostaglandins, including 15-d- $\Delta^{12,14}$ -PGJ₂ and prostaglandin H synthase 2 (COX-2) polyclonal antibody, were from Cayman Chemicals (Ann Arbor, MI). Troglitazone was a gift from Parke Davis (Ann Arbor, MI). Aminoguanidine hemisulfate (AG) and LPS (Serotype 0111:B4) were from Sigma Chemical (St. Louis, MO). Enhanced chemiluminescence reagents were purchased from Amersham (Arlington Heights, IL). Horseradish peroxidase-conjugated donkey anti-rabbit and donkey anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-serum specific for the mouse macrophage iNOS was a gift from Dr. Thomas Misko (G.D. Searle, St. Louis, MO) and mouse anti-rat hsp 70 was from StressGen (Victoria, BC, Canada). All other reagents were from commercially available sources.

CD-1 mouse peritoneal macrophage isolation, islet isolation, and cell culture. Peritoneal macrophages (peritoneal exudate cells [PEC]) were isolated from male CD-1 mice by lavage as previously described (23). After isolation, the cells were plated at a concentration of 400,000 cells/400 μ l complete CMRL-1066 (CMRL-1066 containing 2 mmol/l L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in 24-well microtiter plates and incubated for 3 h in an atmosphere of 95% air and 5% CO₂ at 37°C. Nonadherent cells were removed by washing (three times with complete CMRL) followed by treatment of the adherent macrophages for 30 min with 15-d- $\Delta^{12,14}$ -PGJ₂. Then mouse IFN- γ and LPS were added, and the cells were cultured for the indicated times at 37°C.

RAW 264.7 cells were removed from growth flasks by treatment with 0.05% trypsin-0.02% EDTA at 37°C. Cells were washed two times with complete CMRL-1066, plated at a concentration of 400,000 cells/400 μ l of complete CMRL-1066 in 24-well microtiter plates, and cultured at 37°C for 2–3 h before treatment. The cells were preincubated for 30 min with 15-d- $\Delta^{12,14}$ -PGJ₂ or DMSO. Then mouse IFN- γ and/or LPS were added, and the cells were cultured for the indicated times at 37°C.

Islets from male Sprague Dawley rats were isolated by collagenase digestion as previously described (24). After isolation, the islets were cultured in complete CMRL-1066 in an atmosphere of 95% air and 5% CO₂ at 37°C overnight. Before each experiment, islets were washed in complete CMRL and counted. Experiments were initiated by the addition of 15-d- $\Delta^{12,14}$ -PGJ₂ or DMSO 30 min before addition of LPS and TNF- α , and the islets were cultured at 37°C as indicated.

Western blot analysis. SDS gel electrophoresis and Western blot analysis were performed as previously described (22,25). Dilutions for primary antiserum were as follows: rabbit anti-mouse iNOS, 1:2,000; rabbit anti-COX-2, 1:1,000 (Cayman); rabbit anti-human I κ B, 1:1,500 (Santa Cruz); rabbit anti-phospho-C-Jun NH₂-terminal kinase (JNK), 1:2,000 (New England BioLabs); rabbit anti-JNK, 1:1,000 (Santa Cruz); mouse anti-human hsp 70, 1:1,000 (StressGen). For detection of JNK, phospho-JNK blots were stripped by incubation for 30 min at 50°C in 50 ml of 2% SDS, 100 mmol/l 2-mercaptoethanol, and 62.5 mmol/l Tris-Cl, pH 6.7.

Nitrite and IL-1 determination. Nitrite production was determined by mixing 50 μ l of culture medium with 50 μ l of Griess reagent (26). The absorbance at 540 nm was measured, and nitrite concentrations were calculated from a sodium nitrite standard curve. IL-1 release from mouse PEC was performed using the RINm5F cell IL-1 bioassay as described previously (27,28).

Cell viability. Cell viability was determined by trypan blue exclusion and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium (MTT) oxidation as previously described (29).

Polymerase chain reaction. Total RNA was isolated from islets and macrophages using the Qiagen RNeasy RNA isolation kit, and 11 μ l of each total RNA eluant was used in first-strand cDNA synthesis using the Superscript Preamplication System (Gibco BRL, Life Technologies, Grand Island, NY). A standard 25 μ l polymerase chain reaction (PCR) contained 1 μ l of the reverse transcriptase reaction, 0.2 mmol/l dNTP (Life Technologies), 1.5 mmol/l MgCl₂, 1 \times Assay Buffer B, 1.5 U Taq polymerase (Promega, Madison, WI), 30 pmol forward primer, 30 pmol reverse primer (Life Technologies). IL-1 β , iNOS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed according to the published sequences:

IL-1 β forward 5'-CCTGTGGCCTTGGGCTCAA-3'	PCR product size =
IL-1 β reverse 5'-GGTGCTGATGTACCAGTTGGG-3'	204 bp
iNOS forward 5'-CCAACCGGAGAAGGGGACGAAC-3'	PCR product size =
iNOS reverse 5'-GGAGGTTGGTGGCGCTGGAC-3'	297 bp
GAPDH forward 5'-GCTGGGGCTCACCTGAAGGG-3'	PCR product size =
GAPDH reverse 5'-GGATGACCTTGCCACAGCC-3'	343 bp

Each PCR mixture was overlaid with one drop of molecular grade mineral oil (Sigma) and incubated in a Hybaid Omnigene thermal cycler using the following profile: 94°C for 2 min, 30 cycles of 94°C for 45 s, 55°C for 45 s (60°C for IL-1 β), and 72°C for 75 s, and 30°C for 2 min. One microliter of 10 xylene cyanole loading dye was added to each sample, and 20 μ l was run next to 5 μ l 100-bp ladder (Promega) on 1.5% agarose gels containing 0.1 μ g/ml ethidium bromide.

Northern blot analysis. iNOS mRNA expression by RAW 264.7 and RINm5F cells was determined by Northern blot analysis as previously described (25,28).

RESULTS

15-d- $\Delta^{12,14}$ -PGJ₂ inhibits LPS + IFN- γ -induced nitrite production and iNOS expression. Treatment of RAW 264.7 cells for 24 h with LPS + IFN- γ induces a >20-fold increase in the release of nitrite as compared with untreated cells. In a concentration-dependent manner, 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits LPS + IFN- γ -induced nitrite formation, with half-maximal inhibition at ~0.5 μ g/ml and near complete inhibition at 1 μ g/ml (Fig. 1A). A second PPAR- γ agonist, troglitazone, also inhibits LPS + IFN- γ -induced nitrite production by RAW 264.7 cells, with half-maximal inhibition at ~25 μ g/ml and complete inhibition at 50 μ g/ml. As determined by Western blot analysis, 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits iNOS protein expression in a similar concentration-dependent manner, with complete inhibition observed at 5 μ g/ml (Fig. 1B). Troglitazone at 50 μ g/ml also completely inhibits LPS + IFN- γ -induced iNOS expression by RAW 264.7 cells (data not shown).

Treatment of CD-1 mouse PEC with LPS + IFN- γ stimulates a nearly sixfold increase in nitrite production (Fig. 1C). 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits LPS + IFN- γ -induced nitrite production by mouse PEC, with half-maximal and maximal inhibition at 0.5 and 5 μ g/ml, respectively. Similar to the

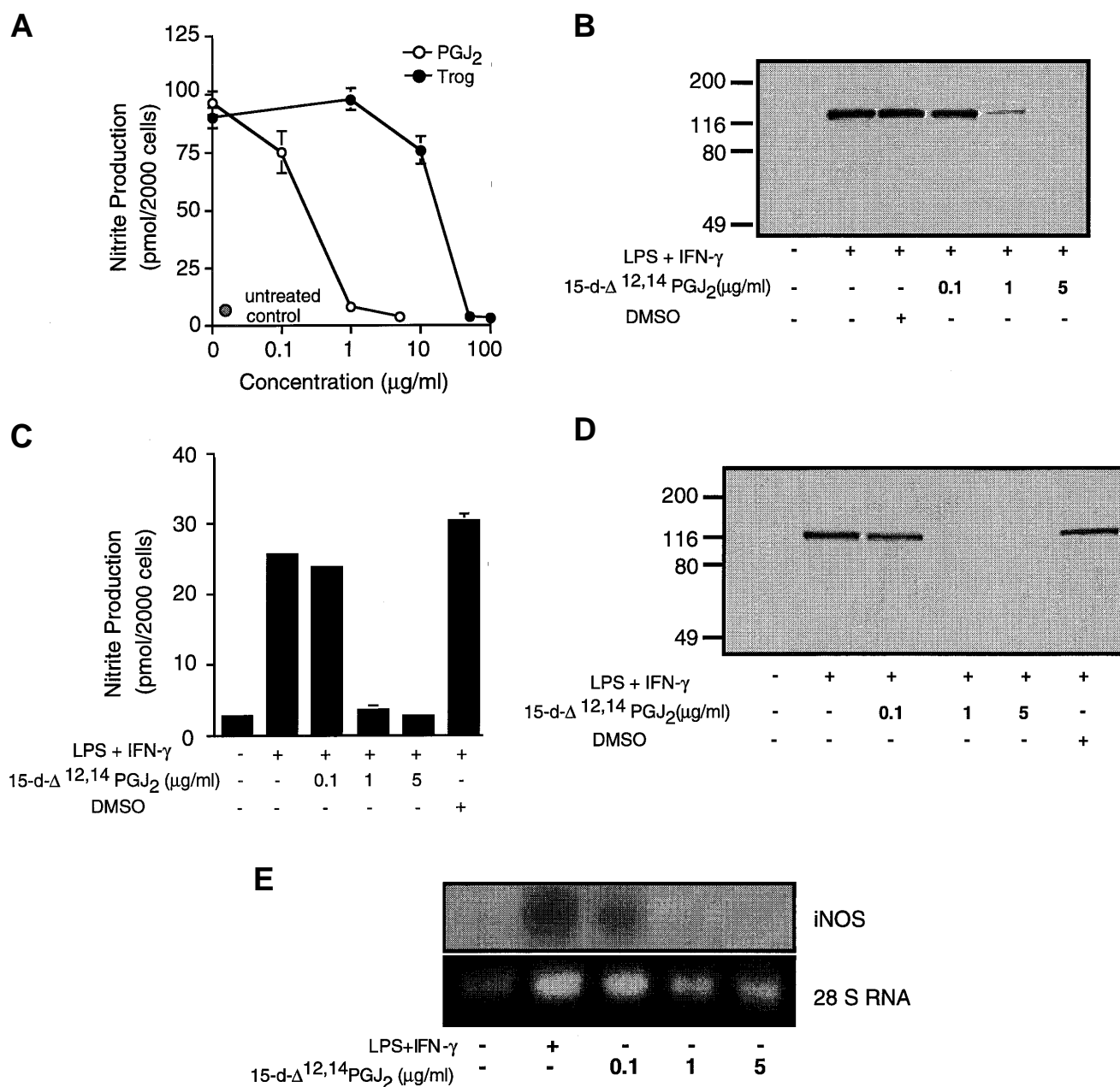


FIG. 1. Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced iNOS expression and nitrite production by macrophages. RAW 264.7 cells (A and B) or CD-1 mouse PEC (C and D) (400,000/400 μ l of complete CMRL-1066) were pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone (Trog) (RAW 264.7 cells only). LPS (10 μ g/ml) and mouse IFN- γ (150 U/ml) were added, and the cells were cultured for 24 h. The supernatant was removed for nitrite determination, and the cells were isolated for Western blot analysis of iNOS expression (B and D). E: Total RNA was isolated from RAW 264.7 cells (5×10^6 /3 ml of complete CMRL-1066) pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ and treated for 4 h with 10 μ g/ml LPS and 150 U/ml mouse IFN- γ . iNOS mRNA accumulation was then determined by Northern analysis. To normalize for RNA loading, the 28S RNA band is also shown. Results for A and C are averages \pm SE of three independent experiments; results for B and D are representative of three independent experiments; and results for E are representative of two independent experiments.

inhibitory effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on nitrite production, at 1 and 5 μ g/ml, 15-d- $\Delta^{12,14}$ -PGJ₂ completely inhibits iNOS expression as determined by Western blot analysis (Fig. 1D). The inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on iNOS mRNA accumulation are shown in Fig. 1E. Treatment of RAW 264.7 cells for 4 h with LPS and IFN- γ results in the accumulation of iNOS mRNA. At 1 and 5 μ g/ml, 15-d- $\Delta^{12,14}$ -PGJ₂ completely inhibits LPS + IFN- γ -induced iNOS mRNA accumulation by RAW 264.7 cells. These findings indicate that 15-d- $\Delta^{12,14}$ -PGJ₂

inhibits NO production by macrophages by preventing LPS + IFN- γ -induced iNOS mRNA accumulation.

Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced iNOS enzymatic activity and cell viability. Results presented in Fig. 1A–E demonstrate that 15-d- $\Delta^{12,14}$ -PGJ₂ is a potent inhibitor of iNOS mRNA and protein expression; however, these experiments do not provide information on the potential inhibitory actions of this prostaglandin on iNOS enzymatic activity. Therefore, a whole cell assay system was

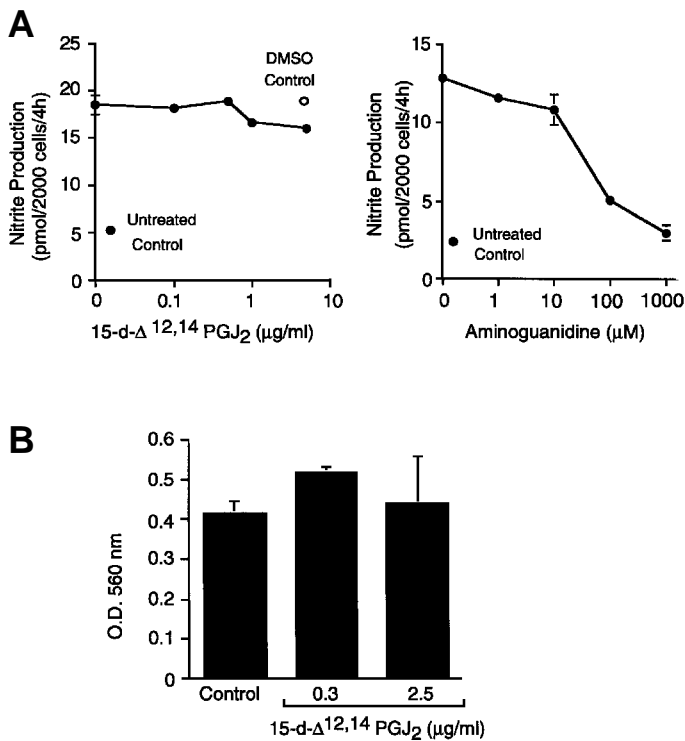


FIG. 2. Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on iNOS enzymatic activity and RAW 264.7 cell viability. A: RAW 264.7 cells (400,000/400 μ l of complete CMRL-1066) were treated for 18 h with 10 μ g/ml LPS and 150 U/ml mouse IFN- γ to stimulate the expression of iNOS. The cells were washed and then incubated for 4 h in 400 μ l of fresh complete CMRL-1066 containing the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ and the iNOS inhibitor AG. The supernatant was removed, and nitrite production was determined. B: RAW 264.7 cells (400,000/400 μ l of complete CMRL-1066) were treated for 18 h with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂. The media was removed, and 1 mg/ml MTT was added. MTT oxidation was determined following a 6-h incubation at 37°C as stated in RESEARCH DESIGN AND METHODS. Results for nitrite are averages \pm SE of four individual experiments, and results for the MTT assay are averages \pm SD of two independent experiments.

used to examine the effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on iNOS enzymatic activity (30). In this assay, RAW 264.7 cells were treated for 18 h with LPS + IFN- γ . The cells were washed three times with complete CMRL-1066 and cultured for an additional 4 h in complete CMRL-1066 containing the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ and the iNOS selective inhibitor aminoguanidine (AG) (30). The culture supernatants were then removed, and nitrite production was determined. As shown in Fig. 2A, 15-d- $\Delta^{12,14}$ -PGJ₂ does not inhibit iNOS enzymatic activity at concentrations ranging from 0.1–5 μ g/ml; however, AG inhibits iNOS enzymatic activity in a concentration-dependent manner, with half-maximal inhibition at ~50 μ mol/l. These findings show that 15-d- $\Delta^{12,14}$ -PGJ₂ is not an inhibitor of iNOS enzymatic activity.

To ensure that the inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on iNOS expression are not associated with macrophage cell death, the effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on RAW 264.7 cell viability were examined. Incubation of RAW 264.7 cells for 18 h with 15-d- $\Delta^{12,14}$ -PGJ₂ at concentrations from 0.1–5 μ g/ml does not change cell viability as determined by trypan blue exclusion (data not shown). The effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on RAW 264.7 cell viability were also assayed by the oxidation of MTT. This assay, which measures mitochondrial dehydrogenase, is a rapid method to determine the overall health of a cell, as increased MTT oxidation is associated with cell proliferation and reduced oxidation is associated with cell death (29). As shown in Fig. 2B, an 18-h incubation with 0.3 or 2.5 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂ does not inhibit the oxidation of MTT by RAW 264.7 cells (Fig. 2B).

Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced IL-1 expression and release from RAW 264.7 cells and primary CD-1 mouse macrophages. In an activated state, macrophages release high levels of the proinflammatory cytokine IL-1. Using the RINm5F cell IL-1 bioassay (27), the effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced IL-1 release by RAW 264.7 cells and CD-1 mouse macrophages were examined. Treatment of RAW 264.7 cells or CD-1 mouse PECs with LPS + IFN- γ results in the release of high levels of

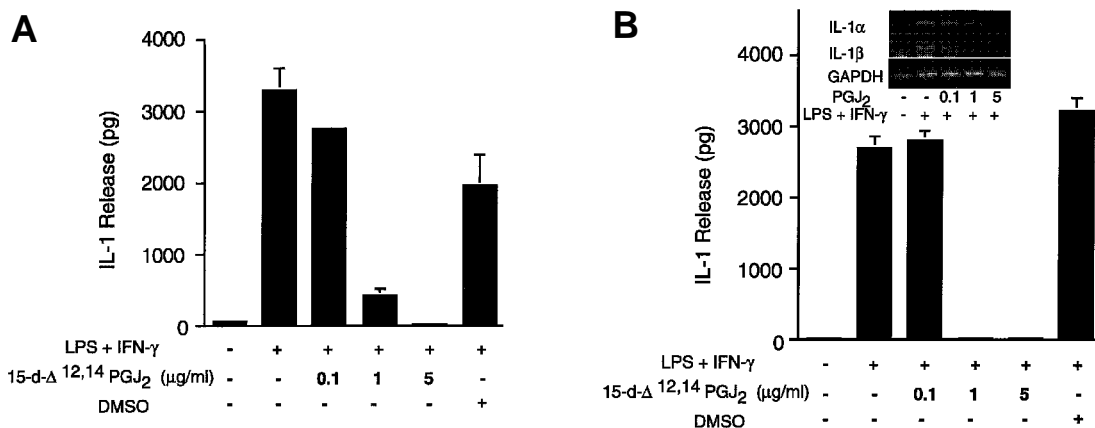


FIG. 3. Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced IL-1 release by RAW 264.7 cells and CD-1 mouse macrophages. RAW 264.7 cells (A) and CD-1 mouse macrophages (B) (400,000/400 μ l of complete CMRL-1066) were pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂. LPS (10 μ g/ml) and mouse IFN- γ (150 U/ml) were added, and the cells were cultured for 24 additional hours. IL-1 release was determined using the RINm5F cell IL-1 bioassay as stated in RESEARCH DESIGN AND METHODS. For the inset in B, CD-1 mouse macrophages (400,000/400 μ l of complete CMRL-1066) were pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂. LPS (10 μ g/ml) and mouse IFN- γ (150 U/ml) were added, and the cells were cultured for 4 additional hours. Total RNA was isolated, and IL-1 α and IL-1 β mRNA accumulation was determined by RT-PCR as stated in RESEARCH DESIGN AND METHODS. As a control, GAPDH mRNA accumulation is also shown. Results for the IL-1 bioassay are averages \pm SE of four independent experiments, and IL-1 α and IL-1 β mRNA accumulation is representative of three independent experiments.

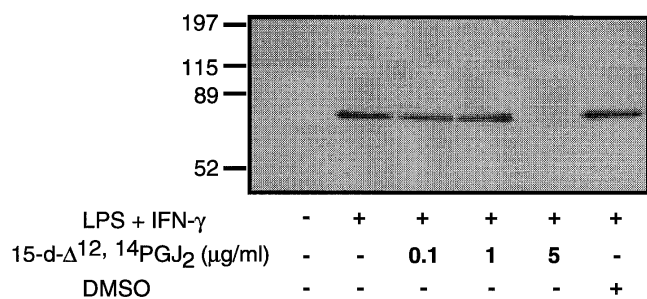


FIG. 4. Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on COX-2 expression by primary CD-1 mouse macrophages. CD-1 mouse macrophages (400,000/400 μ l of complete CMRL-1066) were pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ or DMSO followed by the addition of 10 μ g/ml LPS + 150 U/ml mouse IFN- γ . After a 24-h culture, the cells were isolated, and COX-2 expression was determined by Western blot analysis as stated in RESEARCH DESIGN AND METHODS. Results are representative of two independent experiments.

IL-1 (Fig. 3). In a concentration-dependent manner, 15-d- $\Delta^{12,14}$ -PGJ₂ prevents LPS + IFN- γ -induced IL-1 release from both RAW 264.7 cells and primary mouse peritoneal macrophages, with nearly complete and complete inhibition at 1 and 5 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂, respectively. The inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on IL-1 release appear to reflect an inhibition of LPS + IFN- γ -induced IL-1 expression by CD-1 mouse macrophages. As shown by PCR in Fig. 3B (inset), 1 and 5 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂ attenuates and prevents LPS + IFN- γ -induced IL-1 α and IL-1 β mRNA accumulation, respectively. These findings provide direct evidence that 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits macrophage expression and release of the proinflammatory cytokine IL-1.

Inducible cyclooxygenase expression by CD-1 mouse macrophages is inhibited by 15-d- $\Delta^{12,14}$ -PGJ₂. Expression of the inducible isoform of cyclooxygenase (COX-2) and increased production of prostaglandins are associated with macrophage activation under conditions of injury and inflam-

mation (31). As shown in Fig. 4, treatment of CD-1 mouse PEC with LPS + IFN- γ for 24 h results in COX-2 expression as determined by Western blot analysis. At 5 μ g/ml, 15-d- $\Delta^{12,14}$ -PGJ₂ completely inhibits LPS + IFN- γ -induced COX-2 expression by primary macrophages. In a similar concentration-dependent manner, 15-d- $\Delta^{12,14}$ -PGJ₂ completely inhibits LPS + IFN- γ -induced COX-2 expression by RAW 264.7 cells (data not shown).

Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ derivatives on LPS + IFN- γ -induced nitrite production by RAW 264.7 cells. The inhibitory effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced iNOS expression appear to be specific for the J family of prostaglandins. As shown in Fig. 5, at 1 μ g/ml, prostaglandin (PG) D₂, PGA₁, PGF₂, PGI₂, and PGE₂ do not inhibit LPS-induced nitrite production by RAW 264.7 cells after an 18-h incubation. In addition, at 1 μ g/ml, WY14643 (PPAR- α agonist), docosahexaenoic acid, and linoleic acid also do not modulate LPS-induced nitrite production by RAW 264.7 cells (Fig. 5). However, the PPAR- γ agonists PGJ₂ and Δ^{12} PGJ₂ (at 1 μ g/ml), inhibit LPS-induced nitrite production by RAW 264.7 cells by ~50 and ~95%, respectively. These findings suggest that the J family of prostaglandins—15-d- $\Delta^{12,14}$ -PGJ₂, Δ^{12} PGJ₂, and to a lesser extent PGJ₂—are effective inhibitors of LPS-induced nitrite production by RAW 264.7 cells.

15-d- $\Delta^{12,14}$ -PGJ₂ prevents LPS + TNF- α -induced iNOS and IL-1 β mRNA accumulation in rat islets. Islets contain a small population of resident macrophages (~0.1%, or 10–15 macrophages/2,000 islet cells) that, when activated (in response to TNF + LPS), mediate β -cell damage by the local release of IL-1 in islets, followed by IL-1-induced iNOS expression by β -cells (13,14). In addition, β -cells appear to be the sole islet cellular source of iNOS in response to IL-1 (13). To examine the effectiveness of 15-d- $\Delta^{12,14}$ -PGJ₂ on both islet macrophage activation and β -cell nitric oxide production, the effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on TNF + LPS- and IL-1-induced nitrite production by rat islets were examined. As shown in Fig. 6A, 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits TNF + LPS-induced nitrite formation by rat islets in a concentration-dependent manner, with half-maximal inhibition at ~1 μ g/ml and nearly complete inhibition at 5 μ g/ml. Although 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits TNF + LPS-induced nitrite production by rat islets, this PPAR- γ agonist is much less effective at inhibiting IL-1-induced nitrite production by β -cells: 5 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂ inhibits IL-1-induced nitrite formation by only ~30%. Because TNF + LPS-induced iNOS expression and nitrite production are mediated by the local release of IL-1 β in islets followed by IL-1 β -induced iNOS expression by β -cells (14), the effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on IL-1 β and iNOS mRNA accumulation in rat islets were examined. As shown in Fig. 6B, at 5 μ g/ml, 15-d- $\Delta^{12,14}$ -PGJ₂ nearly completely inhibits TNF + LPS-induced iNOS and IL-1 β mRNA accumulation by rat islets. These results indicate that 15-d- $\Delta^{12,14}$ -PGJ₂ is a potent inhibitor of resident macrophage activation (both iNOS and IL-1 β expression), but is much less effective at inhibiting IL-1-induced iNOS expression by β -cells.

Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on nitrite production and hsp 70 expression by RINm5F cells. Recently, Unger and coworkers (32,33) have shown that the PPAR- γ agonist troglitazone attenuates IL-1-induced nitrite production by islets isolated from both obese and lean Zucker rats and that the reduction in nitrite production is associated with a reduction in islet triglyceride content. Because 15-d- $\Delta^{12,14}$ -PGJ₂ is a PPAR- γ agonist, the potential inhibitory

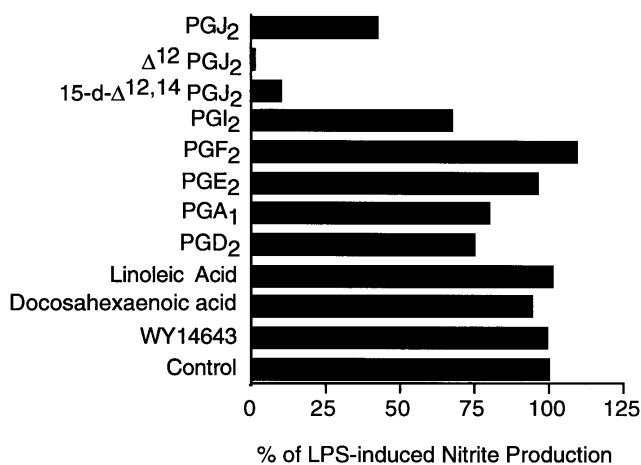


FIG. 5. Effects of prostaglandins on nitrite production by RAW 264.7 cells. RAW 264.7 cells (400,000 cell/400 μ l of complete CMRL-1066) were pretreated for 30 min with 1 μ g/ml of each of the indicated reagents. LPS (10 μ g/ml) was added, and the RAW 264.7 cells were cultured for an additional 18 h. The culture media was removed and nitrite determined as stated in RESEARCH DESIGN AND METHODS. Results are plotted as % of LPS-induced nitrite production and are averages \pm SE of three independent experiments.

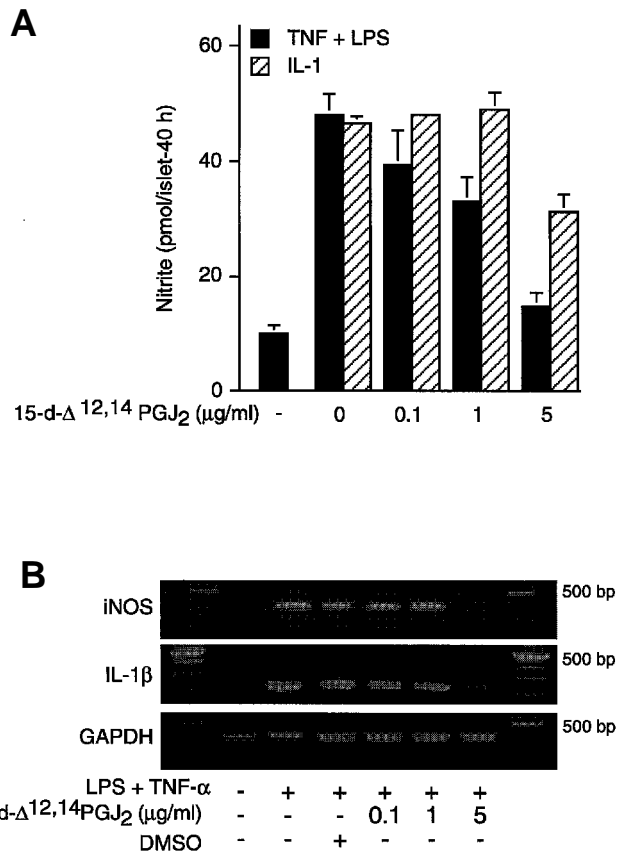


FIG. 6. Effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on TNF + LPS and IL-1-induced nitrite production and iNOS and IL-1 β mRNA accumulation by rat islets. A: Rat islets (120 islets/400 μ l complete CMRL-1066) were pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ or DMSO. LPS (10 μ g/ml) and TNF- α (10 ng/ml) were added, and the islets were cultured for an additional 4 h or 40 h. Nitrite production was determined on the culture supernatants following the 40-h incubation (A), and iNOS and IL-1 β mRNA accumulation was determined by PCR following a 4-h incubation as described in RESEARCH DESIGN AND METHODS (B). Results are representative of three independent experiments.

actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on IL-1-induced nitrite production by β -cells were compared with the actions of troglitazone. Treatment of rat insulinoma RINm5F cells for 24 h with IL-1 results in the expression of iNOS and a 10-fold increase in nitrite production (Fig. 7A and B). In a concentration-dependent manner, 15-d- $\Delta^{12,14}$ -PGJ₂ prevents IL-1-induced iNOS mRNA accumulation and nitrite formation, with half-maximal inhibition at \sim 5 μ g/ml (15 μ mol/l) and complete inhibition at 10 μ g/ml (30 μ mol/l). Troglitazone also prevents IL-1-induced nitrite production by RINm5F cells; however, this PPAR- γ agonist is $>$ 10-fold less effective than 15-d- $\Delta^{12,14}$ -PGJ₂, with a half-maximal inhibition observed at \sim 50 μ g/ml (113 μ mol/l) and complete inhibition at 100 μ g/ml (226 μ mol/l). Troglitazone (100 μ g/ml) also inhibits IL-1-induced iNOS mRNA accumulation by RINm5F cells (data not shown).

We have previously shown that heat shock prevents IL-1- and IL-1 + IFN- γ -induced iNOS expression by rat and human islets, respectively, by preventing cytokine-induced I κ B degradation and NF- κ B nuclear localization (22). In addition, the J prostaglandins have been shown to stimulate the heat shock response in human K562 erythroleukemia cells

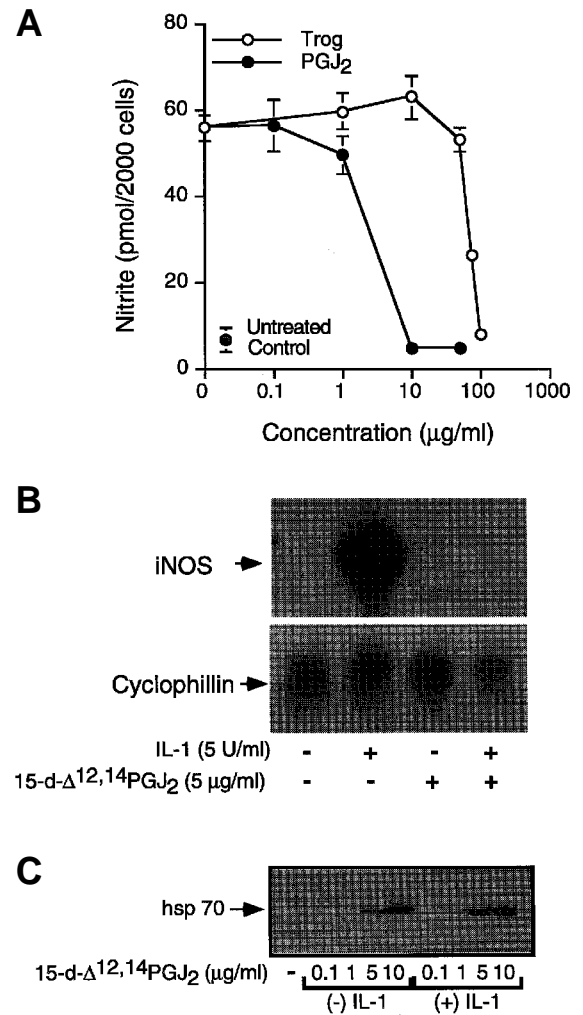


FIG. 7. Concentration-dependent effects of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on IL-1-induced nitrite production and hsp 70 expression by RINm5F cells. RINm5F cells (300,000 cells/400 μ l of complete CMRL-1066) were pretreated for 30 min with the indicated concentrations of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone. IL-1 (5 U/ml) was added, and the cells were cultured for 24 h. The culture media was removed for nitrite determination (A), and hsp 70 expression was determined by Western analysis of the isolated cells (C). B: RINm5F cells (5×10^5 ml of complete CMRL-1066) were pretreated for 30 min with 10 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂. Then IL-1 (5 U/ml) was added, and the cells were cultured for 6 additional hours. The cells were harvested, and total RNA was isolated for Northern blot analysis of iNOS and cyclophilin mRNA (internal control). Results are averages \pm SE of three independent experiments for nitrite and are representative of three experiments for iNOS mRNA accumulation and hsp 70 protein expression.

(20). Therefore, the effects of 15-d- $\Delta^{12,14}$ -PGJ₂ on hsp 70 expression were examined. As shown in Fig. 7C, treatment of RINm5F cells for 24 h with 15-d- $\Delta^{12,14}$ -PGJ₂ results in a concentration-dependent stimulation of hsp 70 expression that is first apparent at 5 μ g/ml—a concentration of this PPAR- γ agonist that provides half-maximal inhibition of IL-1-induced nitrite production by RINm5F cells (Fig. 7A). Importantly, 15-d- $\Delta^{12,14}$ -PGJ₂-induced hsp 70 expression is independent of IL-1, as similar levels of hsp 70 accumulate in the presence or absence of this cytokine. Similar to 15-d- $\Delta^{12,14}$ -PGJ₂, troglitazone also stimulates hsp 70 expression that is first apparent at 50 μ g/ml and maximal at 100 μ g/ml (data not shown).

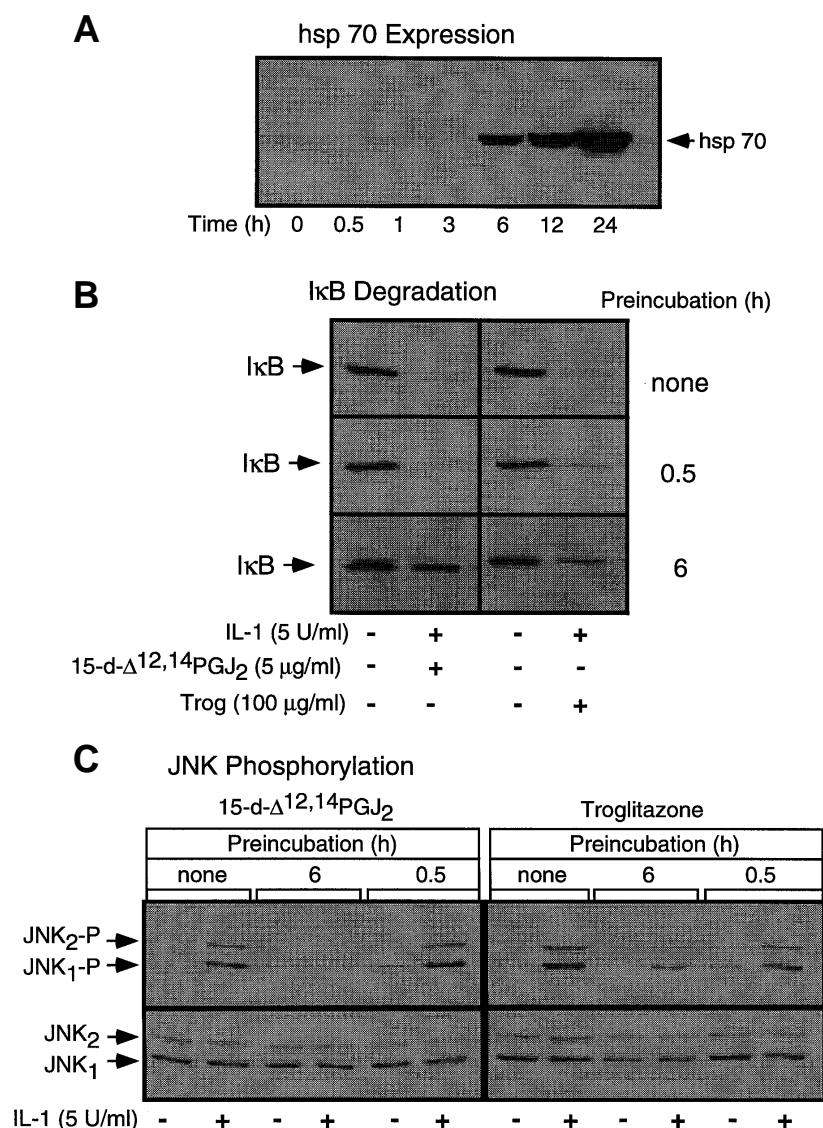


FIG. 8. Time-dependent effects of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on hsp 70 expression and IL-1 signal transduction in RINm5F cells. A: RINm5F cells (300,000 cells/400 µl of complete CMRL-1066) were treated for the indicated times with 5 µg/ml 15-d- $\Delta^{12,14}$ -PGJ₂. The cells were then isolated, and hsp 70 expression was determined by Western blot analysis. B and C: RINm5F cells (300,000 cells/400 µl of complete CMRL-1066) were pretreated for either 30 min or 6 h with 5 µg/ml 15-d- $\Delta^{12,14}$ -PGJ₂ or 100 µg/ml troglitazone as indicated. IL-1 (5 U/ml) was added, and the cells were incubated for 30 min. The cells were then isolated, and I κ B, phospho-JNK, and total JNK were determined by Western blot analysis. Results are representative of two individual experiments.

Time-dependent effects of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on hsp 70 expression, and IL-1-induced I κ B degradation and JNK phosphorylation in RINm5F cells. Because both 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone stimulate hsp 70 expression and because heat shock prevents cytokine signaling (22), the time-dependent effects of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on hsp 70 expression by RINm5F cells were examined by Western blot analysis. As shown in Fig. 8A, 15-d- $\Delta^{12,14}$ -PGJ₂ (5 µg/ml) stimulates the time-dependent expression of hsp 70 that is first apparent at 6 h, and the level of expression increases at 12 and 24 h. In a similar time-dependent manner, 100 µg/ml troglitazone also stimulates hsp 70 expression by RINm5F cells (data not shown). After a 30 min incubation, RINm5F cells fail to express hsp 70 in response to either 15-d- $\Delta^{12,14}$ -PGJ₂ or troglitazone.

To determine if 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone-induced hsp 70 expression correlates with an inhibition of IL-1 signaling events, the effects of these PPAR- γ agonists on IL-1-induced I κ B degradation and JNK phosphorylation in RINm5F cells were examined. Previous studies have shown that I κ B degradation and JNK phosphorylation are time-dependent events that appear to be maximal after a 30-min

incubation of RINm5F cells with IL-1 (22,34–36, and J.A.C., unpublished observations). For these experiments, RINm5F cells were pretreated for either 30 min or 6 h with 5 µg/ml 15-d- $\Delta^{12,14}$ -PGJ₂ or 100 µg/ml troglitazone. IL-1 was then added, and the cells were cultured for an additional 30 min. As shown in Fig. 8B and C, IL-1-induced I κ B degradation and JNK phosphorylation are attenuated if RINm5F cells are preincubated for 6 h with 15-d- $\Delta^{12,14}$ -PGJ₂ or troglitazone, or under conditions in which these PPAR- γ agonists stimulate hsp 70 expression. Importantly, neither 15-d- $\Delta^{12,14}$ -PGJ₂ nor troglitazone inhibit IL-1-induced I κ B degradation or JNK phosphorylation after a 30-min preincubation or under conditions in which these PPAR- γ agonists fail to stimulate hsp 70 expression. These findings suggest that IL-1-induced signaling is substantially diminished in RINm5F cells undergoing the heat shock response (as assessed by hsp 70 expression) in response to these PPAR- γ agonists.

15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone stimulate hsp 70 expression and inhibit IL-1-induced I κ B degradation and JNK phosphorylation in rat islets. To confirm that PPAR- γ agonists stimulate the heat shock response and prevent cytokine-induced IL-1 signaling in β -cells, isolated rat

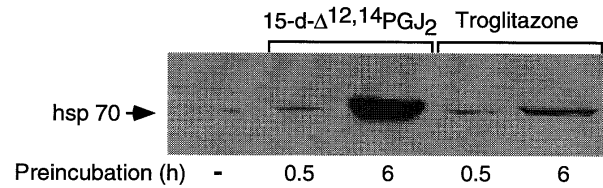
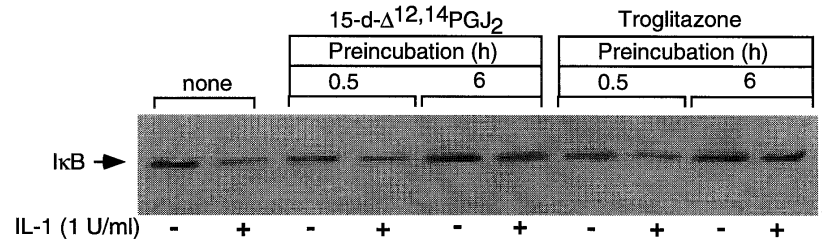
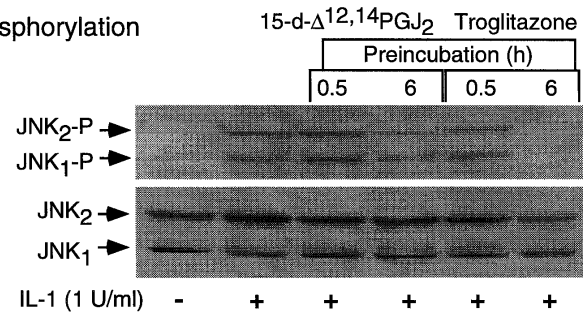
A hsp 70 Expression**B** IκB Degradation**C** JNK Phosphorylation

FIG. 9. Time-dependent effects of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on hsp 70 expression and IL-1 signal transduction in rat islets. **A**: Rat islets (120/400 μ l of complete CMRL-1066) were treated for the indicated times with 10 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂ or 100 μ g/ml troglitazone. The islets were then isolated, and hsp 70 expression was determined by Western blot analysis. **B** and **C**: Rat islets (120/400 μ l of complete CMRL-1066) were pretreated for either 30 min or 6 h with 10 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂ or 100 μ g/ml troglitazone, as indicated. IL-1 (1 U/ml) was added, and the islets were incubated for 30 min. The islets were then isolated, and I κ B, phospho-JNK, and total JNK were determined by Western blot analysis. Results are representative of three individual experiments.

islets were pretreated for 30 min or 6 h with 15-d- $\Delta^{12,14}$ -PGJ₂ or troglitazone. Then IL-1-induced I κ B degradation and JNK phosphorylation was examined. As shown in Fig. 9A, a 6-h incubation of islets with 10 μ g/ml 15-d- $\Delta^{12,14}$ -PGJ₂ stimulates high levels of hsp 70 expression. Troglitazone (100 μ g/ml) also stimulates hsp 70 expression in islets; however, the level of expression is less than the level induced in response to 15-d- $\Delta^{12,14}$ -PGJ₂. Consistent with an inhibition of IL-1 signaling in islets expressing hsp 70 (22), pretreatment of rat islets for 6 h with either troglitazone (100 μ g/ml) or 15-d- $\Delta^{12,14}$ -PGJ₂ (10 μ g/ml) attenuates I κ B degradation and JNK phosphorylation stimulated by a 30-min incubation with IL-1 (Fig. 9B and C). Pretreatment of islets for 30 min with either troglitazone or 15-d- $\Delta^{12,14}$ -PGJ₂ does not inhibit IL-1-induced I κ B degradation or JNK phosphorylation and also does not stimulate the expression of hsp 70 by rat islets (Fig. 9A). Also, a 30-min or 6-h incubation of rat islets with 15-d- $\Delta^{12,14}$ -PGJ₂ or troglitazone does not stimulate either JNK phosphorylation or I κ B degradation (data not shown).

DISCUSSION

In this study, we have identified the J series of prostaglandins as potent anti-inflammatory compounds that prevent cytokine- and endotoxin-induced iNOS, COX-2 and IL-1 expression, nitric oxide production, and IL-1 release by RAW

264.7 cells and CD-1 mouse macrophages. Our findings are consistent with recent reports showing that the inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on macrophage activation correlate with the increased expression of the transcriptional regulator PPAR- γ , and that PPAR- γ activation antagonizes the activation of transcription factors AP-1, STAT, and NF- κ B (17,18,21). Recently, it has been reported that 15-d- $\Delta^{12,14}$ -PGJ₂ is an inhibitor of iNOS enzymatic activity, in addition to iNOS expression (21). Importantly, the inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on LPS + IFN- γ -induced nitrite production by mouse macrophages do not appear to be associated with the inhibition of iNOS enzymatic activity. Using a whole cell iNOS enzymatic activity assay, we show that 15-d- $\Delta^{12,14}$ -PGJ₂ does not inhibit the enzymatic activity of iNOS, whereas the iNOS selective inhibitor AG prevents iNOS activity with an inhibitory constant (IC₅₀) of ~50 μ mol/l (30) (Fig. 2).

In addition to the inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on peritoneal macrophages, we also present evidence that this prostaglandin prevents resident macrophage activation. Previous studies have shown that treatment of rat and human islets with TNF + LPS or TNF + LPS + IFN- γ , respectively, results in the expression of iNOS and the inhibition of insulin secretion (13–15). The inhibitory actions of these cytokines on insulin secretion and stimulatory actions on iNOS expression are completely prevented by the IL-1 receptor antagonist

protein. These findings indicate that intra-islet production of IL-1 followed by IL-1-induced iNOS expression by β -cells results in the inhibition of β -cell function (14). Resident islet macrophages have been confirmed as the islet cellular source of IL-1 in response to TNF + LPS. IL-1 β appears to be the primary isoform of IL-1 expressed and released by activated resident islet macrophages (14). In the current study, we show that 15-d- $\Delta^{12,14}$ -PGJ₂ is a potent inhibitor of TNF + LPS-induced IL-1 β and iNOS mRNA expression and of nitrite production by isolated rat islets. Also, 15-d- $\Delta^{12,14}$ -PGJ₂ appears to be ~10-fold more effective at inhibiting resident macrophage activation in response to TNF- α + LPS as compared with the inhibitory actions of this PPAR- γ agonist on IL-1-induced nitrite production by β -cells. These findings provide direct evidence that 15-d- $\Delta^{12,14}$ -PGJ₂ is an effective inhibitor of both resident and peritoneal macrophage activation. In addition, these studies show a clear concentration-dependent difference in the responsiveness of β -cells and macrophages to 15-d- $\Delta^{12,14}$ -PGJ₂, wherein β -cells are >10-fold less responsive than macrophages. It is possible that the difference in inhibitory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ on iNOS expression may be explained by the level of PPAR- γ expressed in β -cells and macrophages, an issue we are currently evaluating.

Recently, troglitazone has been shown to reduce free fatty acid- and IL-1-induced nitrite production by islets isolated from lean and fatty Zucker rats (33). In addition to inhibiting nitric oxide production, troglitazone also reduces islet triglyceride content, suggesting that reductions in triglyceride levels protect islets from the damaging actions of IL-1 and nitric oxide (32). Consistent with these studies, we show that troglitazone at 100 μ g/ml inhibits IL-1-induced nitrite production and iNOS expression by insulinoma RINm5F cells, although troglitazone is ~10-fold less effective than 15-d- $\Delta^{12,14}$ -PGJ₂. Taken together, these studies provide two mechanisms by which troglitazone may attenuate IL-1-induced nitric oxide production by β -cells: 1) reduction in islet triglyceride levels resulting in a reduced sensitivity of islets to IL-1 (32,33); and/or 2) direct inhibition of IL-1-induced iNOS expression, independent of the actions of increased triglyceride levels.

Recently, evidence has implicated the heat shock response as one mechanism by which β -cells are protected from the damaging actions of IL-1 on islet function and viability. Overexpression of hsp 70 in RINm5F cells prevents nitric oxide-induced cell lysis (37), and liposomal delivery of hsp 70 prevents the damaging actions of IL-1 on islet function (38). We have shown that the inhibitory actions of IL-1 on insulin secretion and mitochondrial aconitase activity and the stimulatory effects on iNOS expression are prevented if islets are heat shocked for 60 min at 42°C before cytokine stimulation (22). The protective actions of heat shock on islet function are associated with an inhibition of IL-1-induced I κ B degradation and NF- κ B nuclear localization resulting in the inhibition of iNOS expression (22). In this study, we show that 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone stimulate hsp 70 expression by the β -cell line RINm5F in a time-dependent fashion that is first apparent after a 6-h incubation. A 6-h incubation with 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone also stimulates hsp 70 expression by rat islets. The stimulatory actions of 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone on hsp 70 expression are associated with the inhibition of signaling events stimulated

by IL-1. These signaling events include IL-1-induced I κ B degradation and JNK phosphorylation. Under conditions in which troglitazone and 15-d- $\Delta^{12,14}$ -PGJ₂ fail to stimulate hsp 70 expression (after a 30-min preincubation), IL-1 stimulates JNK phosphorylation and I κ B degradation; however, after a 6-h pretreatment with these PPAR- γ agonists (or under conditions in which 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone stimulate hsp 70 expression), IL-1 fails to stimulate either JNK phosphorylation or I κ B degradation in RINm5F cells and rat islets. Importantly, these findings provide evidence that prolonged exposures of RINm5F cells to PPAR- γ agonists result in induction of the heat shock response (as evidenced by hsp 70 expression) and impairment of cytokine signaling. It is not clear if hsp 70 mediates the impairment in IL-1 signaling induced by PPAR- γ agonists or if other stress response proteins also participate. Interestingly, 15-d- $\Delta^{12,14}$ -PGJ₂ has been shown to stimulate heme oxygenase-1 expression by RAW 264.7 cells (21), and heme oxygenase expression appears to protect islets from the damaging effects of IL-1 (39). In conclusion, the findings presented in this study indicate that the PPAR- γ agonists 15-d- $\Delta^{12,14}$ -PGJ₂ and troglitazone are potent anti-inflammatory molecules that 1) prevent cytokine-induced inflammatory gene expression; and 2) prevent cytokine signaling by a mechanism that is associated with induction of the heat shock response.

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