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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits IL-1β–induced cyclooxygenase-2 expression in mesangial cells

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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits IL-1β–induced cyclooxygenase-2 expression in mesangial cells.

Background. Cyclooxygenase-2 (COX-2), a key enzyme in the synthesis of prostaglandins, is induced in mesangial cells in response to proinflammatory cytokines. Recently, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), one of the natural ligands of peroxisome proliferator-activated receptor γ (PPARγ), has been reported to have an anti-inflammatory effect. Therefore, we examined the effect of 15d-PGJ_2 on COX-2 expression in cultured rat mesangial cells.

Methods. Mesangial cells were incubated with 15d-PGJ_2 for 30 minutes and then exposed to interleukin-1β (IL-1β). The expression of COX-2 mRNA and proteins was determined by Northern blot and immunoblot analyses, respectively. Accumulation of prostaglandin E_2 (PGE_2) was measured by an enzyme-linked immunosorbent assay (ELISA). Activities of mitogen-activated protein kinases (MAPKs) were evaluated by an immunoblot analysis. DNA binding activities of activator protein-1 (AP-1) or nuclear factor-κB (NF-κB) were examined by an electrophoretic mobility shift assay (EMSA). The activities of PPAR responsive elements (PPRE) and COX-2 promoter were measured by a luciferase reporter assay.

Results. 15D-PGJ_2 significantly suppressed IL-1β–induced COX-2 expression and PGE_2 production, but thiazolidinediones, synthetic PPARγ ligands, did not affect COX-2 expression. Moreover, the cells transfected with a PPRE luciferase reporter did not respond to 15d-PGJ_2. IL-1β rapidly activated extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), which were involved in the up-regulation of COX-2 induction, but 15d-PGJ_2 inhibited the activation of these kinases. 15d-PGJ_2 inhibited the IL-1β–induced increase in binding activities of nuclear proteins to consensus AP-1 site and AP-1–like site of COX-2 promoter but not of NF-κB. IL-1β was unable to activate the COX-2 promoter when the AP-1–like site was mutated.

Conclusions. These data suggest that 15d-PGJ_2 inhibits IL-1β–induced COX-2 expression, independent of PPARγ activation, by suppression of ERK and JNK pathways and AP-1 activation in mesangial cells. Thus, 15d-PGJ_2 may play an important role in the negative feedback mechanism of COX-2 expression in renal inflammation and may be useful as an anti-inflammatory agent.

Key words: 15d-PGJ_2, COX-2, mitogen-activated protein kinases, activator protein-1, glomerular mesangial cells, anti-inflammatory.

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Glomerular mesangial hyperplasia is one of the pathological characters in various glomerular diseases. On activation, mesangial cells are able to proliferate, produce extracellular matrix proteins, and release numerous cytokines, growth factors, and prostaglandins [1, 2], suggesting that mesangial cells play an important role in the process of glomerular damage.

Interleukin-1β (IL-1β) is an immunoregulatory and proinflammatory cytokine released by various cells in response to diverse extracellular stimuli, such as infection, inflammation, cell injury, and toxins [3]. IL-1β, produced by both infiltrating macrophages and activated mesangial cells in glomerulonephritis, is able to stimulate the production of prostaglandins and nitric oxide (NO) in mesangial cells [4, 5]. Prostaglandins are arachidonic acid metabolites that influence various cellular functions, including inflammation and cell proliferation [6], suggesting that prostaglandins induced by IL-1β may contribute to the process of glomerular injury. Cyclooxygenase (COX) is a key enzyme that catalyzes oxygenation in prostaglandin synthesis. COX converts arachidonic acid to prostaglandin G_2 (PGG_2) and subsequently reduces PGG_2 to prostaglandin H_2 (PGH_2), which is further metabolized to the biologically active prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TXA_2) by their respective synthases [6, 7]. Two COX isoforms, COX-1 and COX-2, have been identified [8]. COX-1 is a constitutive form and present in various types of cells and tissues. COX-2 is an inducible form, undetectable at baseline in normal tissues, and expressed by various stimuli, such as inflammatory cytokines and growth factors, followed by the overproduction of prostanoids [9]. In mesangial cells, COX-2 was shown to be rapidly induced by IL-1β [10, 11], serotonin [12], lipopolysaccharide (LPS) [13], platelet-derived growth factor [14], and endothelin [15, 16].

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of a nuclear hormone receptor superfamily of ligand dependent transcription factors [17]. PPARγ forms a heterodimer with retinoid X receptor and the complex bind to a PPAR responsive element (PPRE) in the promoter of target genes [17]. PPARγ is expressed at high levels in adipose tissue and is a critical regulator of adipocyte differentiation and glucose metabolism [18]. PPARγ is activated by synthetic ligands, thiazolidinediones [19] (such as troglitazone and pioglitazone), and by a potent natural ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [20, 21]. 15D-PGJ_2 is a natural metabolite derived from PGD_2, which is the most abundant prostaglandin in normal tissues, and has the highest binding affinity to PPARγ in the J-series prostaglandins [22]. 15D-PGJ_2 has been shown to inhibit the production of inflammatory cytokines and inducible nitric oxide synthase (iNOS) in activated peripheral monocytes and macrophages [23, 24]. The anti-inflammatory effect of 15d-PGJ_2 also has been found even in the non-immune cells such as vascular smooth muscle cells [25, 26] and endothelial cells [27].

Although several studies have shown that the anti-inflammatory effect of 15d-PGJ_2 appears to be regulated through transcriptional inhibition by a PPARγ-dependent mechanism [23, 24, 28], recent evidence suggests that the function of 15d-PGJ_2 also is regulated by a PPARγ-independent pathway [29–33]. In fact, 15d-PGJ_2 is able to suppress the cytokine production in PPARγ-deficient macrophage [29] and to block LPS-induced iNOS expression by a mechanism independent of PPARγ in microglial cell lines [31]. Furthermore, nuclear factor-κB (NF-κB), a well-known inflammatory transcription factor, is repressed by 15d-PGJ_2 in a PPARγ-independent manner [32, 33].

Therefore, we hypothesized that 15d-PGJ_2 has an anti-inflammatory action in mesangial cells. To prove this hypothesis, we examined the effect of 15d-PGJ_2 on IL-1β–induced COX-2 expression and PGE_2 production in rat glomerular mesangial cells. We found that 15d-PGJ_2 partially inhibits IL-1β–induced COX-2 expression through the suppression of the MAPKs and AP-1 pathways. To our knowledge, the results of our study provide the first experimental evidence that 15d-PGJ_2 has an anti-inflammatory action via a PPARγ-independent mechanism in mesangial cells.

METHODS

Materials

Recombinant rat IL-1β was obtained from Sigma (St. Louis, MO, USA). 15d-PGJ_2 was bought from Calbiochem (La Jolla, CA, USA). Troglitazone and Pioglitazone were kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan), respectively. Anti-COX-2 polyclonal antibody and prostaglandin E_2 EIA kits were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-phospho p42/p44 ERK, anti-phospho p38 MAPK and anti IκB-α antibodies, and PD 98059 were bought from New England Biolabs (Beverly, MA, USA). Consensus oligonucleotides of AP-1 and NF-κB, anti-phospho JNK antibody, and U 0126 were purchased from Promega (Madison, WI, USA). Anti-ERK 2, JNK 1, and p38 MAPK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [α-^{32}P] dCTP and [γ-^{32}P] ATP were bought from New England Nuclear (Boston, MA, USA). Rat COX-2 cDNA (bases 1229-1813) was prepared by reverse transcription with the polymerase chain reaction (RT-PCR) from mRNA of rat kidney as previously described [34], and the cDNA fragment was subcloned into pCR II vector with a TA cloning kit (Invitrogen, Groningen, Netherlands). All other reagents were of chemical grade and purchased from standard suppliers.

Mesangial cell culture and experimental procedure

Glomerular mesangial cells were obtained from a culture of glomeruli that were isolated from six-week-old male Sprague-Dawley rats by a sieving method as previously described [35]. The isolated glomeruli were cultured in RPMI 1640 medium containing 20% heat-inactive fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenious acid. Cultured cells were identified as mesangial cells by morphological and biochemical characters as previously described [36]. Cultured mesangial cells from the 4th to 10th passages were used for the experiments. Subconfluent mesangial cells were made quiescent by incubating with serum-free RPMI 1640 medium supplemented with 0.2% FFA free-bovine serum albumin (BSA) for 48 hours. Quiescent cells were incubated with various concentrations of IL-1β in an experimental medium (RPMI 1640 medium with 0.2% BSA and 20 mmol/L HEPES, pH 7.4) for the indicated time intervals at 37°C. For the experiments with 15d-PGJ_2, the cells were incubated with the indicated concentrations of 15d-PGJ_2 for 30 minutes at 37°C prior to their exposure to IL-1β. After the incubation, the cells were harvested for the determination listed below.

Northern blot analysis

Total RNA (12 μg) was isolated by guanidium and phenol extraction (TRIzol Reagent; Gibco BRL, Grand Island, NY, USA), electrophoresed on 1% formaldehyde-agarose gels, and transferred onto a nylon membrane (Nytran; Schleider & Schuell, Dassel, Germany). Rat COX-2 cDNA was labeled with [α-^{32}P] dCTP by a random primer labeling method (Bca BEST; Takara, Shiga, Japan). The membranes were hybridized with rat

COX-2 cDNA in hybridization buffer [0.5 mol/L $NaPO_4$, pH 7.0, 1% BSA, 7% sodium dodecyl sulfate (SDS), and 1 mmol/L ethylenediaminetetraacetic acid (EDTA)] at 65°C for 16 hours. The membranes were autoradiographed with screens at −80°C overnight and rehybridized with a radioactive probe of acidic ribosomal phosphoprotein PO (36B4) cDNA as an internal standard [37].

Immunoblot analysis

Mesangial cells were rinsed twice with ice-cold PBS and lysed in 400 μL ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40. The cell lysates were centrifuged at 15,000 rpm for 10 minutes after sonication and the supernatants were collected. The concentrations of the protein in the supernatants were measured by the Bradford method (Bio-Rad, Hercules, CA, USA) and the cell lysates containing 40 μg protein were boiled in SDS sample buffer [62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 50 mmol/L dithiothreitol (DTT), 0.1% bromophenol blue] for five minutes. The samples were electrophoresed on 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA). After blocking with TBS-T (Tris buffered saline containing 0.1% Tween 20) containing 5% nonfat milk, the membranes were incubated with 1:1000 diluted anti-COX-2 polyclonal antibody or anti-IκBα antibody at 4°C overnight. The membranes were washed three times and incubated with 1:1000 diluted horseradish peroxide (HRP) conjugated-anti-rabbit secondary antibody (Amersham, Buckinghamshire, UK) at room temperature for one hour. The immunoreactive bands were detected with an enhanced chemiluminescence (ECL) detection system (NEN Life Science Products, Boston, MA, USA).

Measurement of prostaglandin E_2 levels

The culture medium of mesangial cells on a six-well plate was collected and stored at −80°C until assay. Accumulation of PGE_2 in the medium was measured with a PGE_2 enzyme-linked immunosorbent assay (ELISA) kit. The concentrations of PGE_2 were corrected with the total amount of cellular proteins.

Reverse transcription-polymerase chain reaction (RT-PCR)

For RT-PCR analysis, total RNA (200 ng) was reverse transcribed with random hexamers using the Gene-Amp RNA-PCR kit (Perkin-Elmer, Branchburg, NJ, USA) following the manufacturer's directions. The cycling conditions were 30 cycles of 15 seconds at 94°C, 30 seconds at 58°C, and 45 seconds at 72°C. The primers for rat PPARγ were 5′-AACCGGAACAAATGCCAGTA-3′ and 5′-TGGCAGCAGTGGAAGAATCG-3′, and then amplified a 386 bp fragment [26]. The primers for GAPDH, used as control, were 5′-GGTCGGTGTCAACGGAT TTG-3′ and 5′-GGCATGTCAGATCCACAACG-3′, and then amplified a 728 bp fragment. The resulting products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Plasmids

The AOx-TK-luciferase reporter construct containing three PPAR-responsive elements (PPRE3-TK-luciferase) and PPARγ expression plasmid were generously provided by Dr. Christopher K. Glass (University of California) [24]. To construct a reporter of the rat COX-2 promoter, a fragment of rat COX-2 promoter (bases −373/+24) was amplified by polymerase chain reaction (PCR) with the use of rat genomic DNA as a template, and the product was subcloned into a luciferase expression vector, PGL2 basic (Promega) at MluI-XhoI sites, as previously described [16]. Since one AP-1–like region was detected on the rat COX-2 promoter [38], a mutation of this AP-1–like site was made by a PCR based site directed mutagenesis (Stratagene, La Jolla, CA, USA) using a pair of oligonucleotides (−173/−147) substituted CTCATTTGCGTGA**GTAA**AGCCTGCCCC for CTC ATTTGCGTGA**CCTG**AGCCTGCCCC. The sequence authenticity of these plasmids was confirmed by a rhodamine terminator cycle sequence system (ABI) and an ABI PRISM 310 genetic analyzer (Foster City, CA, USA).

Transient transfection and luciferase assay

When the mesangial cells were subconfluent on a six-well plate, the medium was replaced with serum-free RPMI 1640. The cells were then transfected with 0.3 μg of the PPRE3-TK-luciferase plasmid reporter (PPRE-luc), 0.1 μg of the pCMV-Lac-Z plasmid, and either 0.8 μg of the PPARγ expression vector or a control vector (pcDNA3; Invitrogen) by LipofectAMINE-plus reagents (Gibco-BRL, Rockville, MD, USA). Three hours after transfection, the cells were starved in RPMI 1640 with 0.2% BSA for 24 hours followed by the stimulation with 15d-PGJ_2 for 16 hours. After washing twice with cold phosphate-buffered saline (PBS), cells were harvested in 200 μL of a reporter lysis buffer (Promega). Twenty microliter aliquots of extracts were used to measure luciferase activity by Luciferase Assay System (Promega) and a luminometer (Auto LUMIcounter Nu1422ES; Nition, Tokyo, Japan). Co-transfected β-galactosidase activity was determined to normalize the luciferase activity.

Similarly, cells were transfected with either 0.8 μg of the pGL2-COX2 or pGL2-COX2–mutant plasmid reporter gene. The cells were incubated with or without 10 μmol/L 15d-PGJ_2 for 30 minutes and then stimulated with IL-1β for 24 hours. Luciferase and β-galactosidase activities were determined as described above.

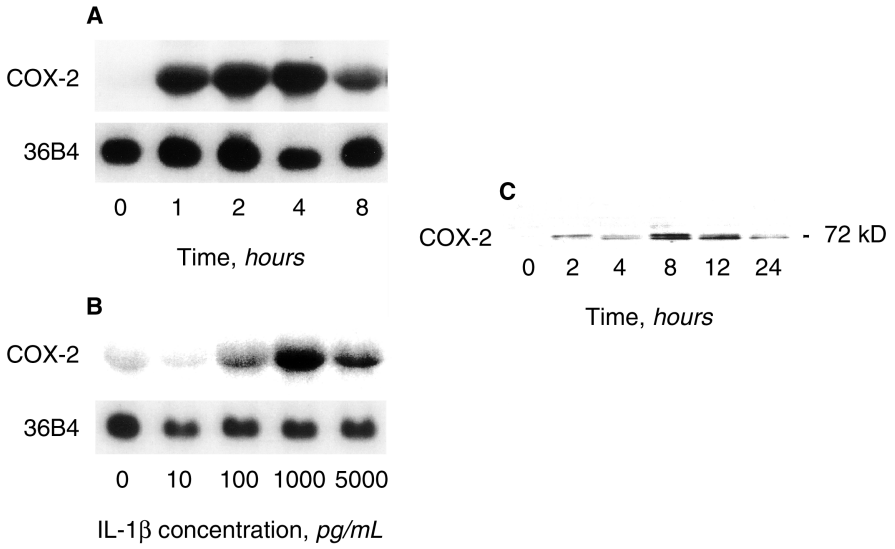

Fig. 1. Interleukin-1β (IL-1β) induces cyclooxygenase-2 (COX-2) expression in mesangial cells. (*A*) Time course of the COX-2 mRNA induction. Mesangial cells were incubated with IL-1β (1000 pg/mL) for the indicated time periods. Isolated total RNA (12 μg) was subjected to Northern blot analysis and hybridized with COX-2 and 36B4 cDNA as an internal control. (*B*) Dose dependency of IL-1β–induced COX-2 mRNA expression. Mesangial cells were treated with various concentrations of IL-1β from 10 pg/mL to 5000 pg/mL for 2 hours. (*C*) Time course of the COX-2 protein induction. Mesangial cells were incubated with IL-1β (1000 pg/mL) for the indicated time periods. Whole cell lysates were subjected to immunoblot analysis with anti-COX-2 antibody. The results are representative of five independent experiments that yielded similar results.

Measurement of the activities of ERK, JNK and p38 MAPK

Activities of ERK, JNK, and p38 MAPK were determined by measuring the levels of phosphorylation of each kinase by immunoblot analysis. For the assay, mesangial cell lysates were electrophoresed on 12% SDS-polyacrylamide gels and electrotransferred to PVDF membranes. After blocking with TBS-T and 5% BSA, the membranes were probed with anti-phospho ERK, phospho JNK, or phospho p38 MAPK antibodies as described above. The membranes were reprobed with anti-ERK2, JNK1, or p38 MAPK antibodies as an internal control.

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Mesangial cells were washed twice with ice-cold PBS and lysed by addition of a hypotonic buffer (10 mmol/L HEPES-KCl, pH 7.9, 1 mmol/L EDTA, 15 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L DTT and protease inhibitor cocktail; Boehringer Mannheim, Lewes, UK) with 0.8% Nonidet P-40, and the lysates were centrifuged at 6000 rpm for 10 minutes. The pellets were resuspended in high salt buffer (hypotonic buffer with 420 mmol/L NaCl and 25% glycerol), rotated for 30 minutes at 4°C and centrifuged at 15,000 rpm for 30 minutes. The supernatants were used as nuclear extracts and the samples were frozen immediately in liquid nitrogen and stored at −80°C until use. EMSAs were performed by incubating the nuclear proteins (4 μg) with 1 μg poly (dI-dC) in binding buffer (10 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, and 5% glycerol) at room temperature for 20 minutes and then reacted with [γ-^{32}P] ATP-end-labeled AP-1 or NF-κB consensus oligonucleotide, or COX-2 promoter oligonucleotide containing the AP-1–like site at room temperature for 30 minutes. The reaction mixtures were electrophoresed on a 4% polyacrylamide gel and autoradiographed.

Statistical analysis

The data were expressed as means ± SD. Analysis of variance (ANOVA) followed by Scheffe's test was used to determine significant difference in multiple comparisons and statistical significance was defined as $P < 0.05$.

RESULTS

Effect of 15d-PGJ$_2$ on IL-1β–induced COX-2 expression and PGE$_2$ production

We first examined IL-1β–induced expression of COX-2 in mesangial cells. IL-1β induced COX-2 mRNA expression after one hour with a maximal stimulation at four hours (Fig. 1A). IL-1β was able to induce COX-2 mRNA in a dose-dependent manner with a maximal stimulation at 1000 pg/mL (Fig. 1B). COX-2 protein synthesis also was stimulated by IL-1β with a maximum level at eight hours (Fig. 1C).

To evaluate the effect of 15d-PGJ$_2$ on COX-2 expression, the cells were incubated with 15d-PGJ$_2$ for 30 minutes prior to the stimulation with IL-1β. The treatment with 15d-PGJ$_2$ significantly suppressed IL-1β–induced COX-2 mRNA expression in a dose-dependent manner (Fig. 2A). Furthermore, IL-1β–induced expression of COX-2 protein was partially inhibited by the treatment with 10 μmol/L 15d-PGJ$_2$ (Fig. 2 B, C). In contrast, the synthetic ligands of PPARγ, troglitazone (50 μmol/L) and pioglitazone (10 μmol/L), were unable to inhibit IL-1β–induced COX-2 mRNA expression (Fig. 2D) or protein production (Fig. 2E). These results suggest that 15d-PGJ$_2$ may act by a different mechanism from thiazolidinediones in mesangial cells.

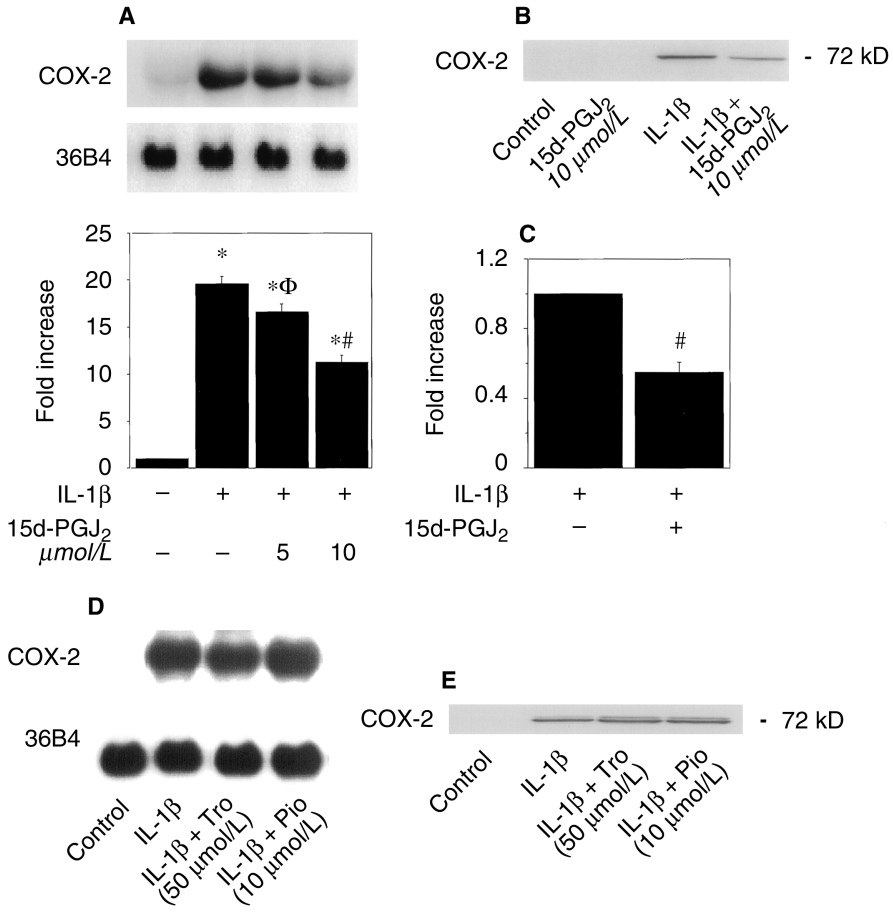

Fig. 2. 15Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15D-PGJ$_2$) inhibits interleukin-1β (IL-1β)–induced COX-2 expression. (*A*) The cells were incubated in the presence or absence of 15d-PGJ$_2$ for 30 minutes and stimulated with 1000 pg/mL IL-1β for 4 hours. Total RNA (12 μg) was subjected to Northern blot analysis and hybridized with COX-2 and 36B4 cDNA as an internal control. The results of a densitometric analysis of five independent experiments are shown. *$P < 0.01$ vs. control, Φ$P < 0.05$ vs. IL-1β, #$P < 0.01$ vs. IL-1β. (*B*) The cells were incubated in the presence or absence of 10 μmol/L 15d-PGJ$_2$ for 30 minutes and stimulated with 1000 pg/mL IL-1β for 8 hours. Whole cell lysates were subjected to immunoblot analysis with anti-COX-2 antibody. (*C*) The results of a densitometric analysis of five independent immunoblot experiments are shown. #$P < 0.01$ vs. IL-1β. (*D*) The cells were pretreated with 50 μmol/L troglitazone (Tro) or 10 μmol/L pioglitazone (Pio) for 30 minutes and stimulated with 1000 pg/mL IL-1β for 4 hours. Total RNA was subjected to Northern blot analysis. (*E*) The cells were pretreated with 50 μmol/L troglitazone (Tro) or 10 μmol/L pioglitazone (Pio) for 30 minutes and stimulated with 1000 pg/mL IL-1β for 8 hours. Whole cell lysates of rat mesangial cells were subjected to immunoblot analysis with anti-COX-2 antibody. The results are representative of five independent experiments that yielded similar results.

Next, the effect of 15d-PGJ$_2$ on IL-1β-induced production of PGE$_2$, which is one of the major products of COX-2, was examined. PGE$_2$ accumulation in the medium increased about 15-fold by the stimulation of IL-1β for 24 hours. Similarly to the effect on COX-2 expression, 15d-PGJ$_2$ significantly inhibited IL-1β–induced production of PGE$_2$ in a dose-dependent manner (Fig. 3).

Effect of 15d-PGJ$_2$ on PPARγ activation

To determine whether 15d-PGJ$_2$ was able to activate PPARγ, we first examined the expression of PPARγ mRNA in rat mesangial cells. Although RT-PCR revealed the 386 bp band, which corresponded to PPARγ mRNA in rat mesangial cells, both IL-1β and 15d-PGJ$_2$ failed to affect its expression (Fig. 4A). Moreover, we found that in mesangial cells transfected with PPRE-luc alone, 15d-PGJ$_2$ failed to stimulate the reporter activity in concentrations used in the present study (Fig. 4B). However, the overexpression of PPARγ increased basal PPRE reporter activity and restored the ability of 15d-PGJ$_2$ to induce it.

Effect of 15d-PGJ$_2$ on activation of NF-κB

The transcription factor NF-κB has been reported to play a role in the regulation of COX-2 gene expression

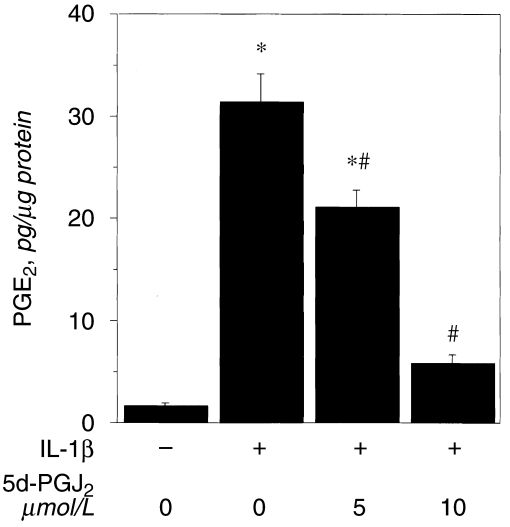

Fig. 3. 15D-PGJ$_2$ attenuates prostaglandin E$_2$ (PGE$_2$) accumulation in response to IL-1β. The cells were incubated in the presence or absence of 15d-PGJ$_2$ for 30 minutes and stimulated with 1000 pg/mL IL-1β for 24 hours. The culture media were collected and analyzed for PGE$_2$ content. The data shown are mean ± SD of five independent experiments that yielded similar results. *$P < 0.01$ vs. control, #$P < 0.01$ vs. IL-1β.

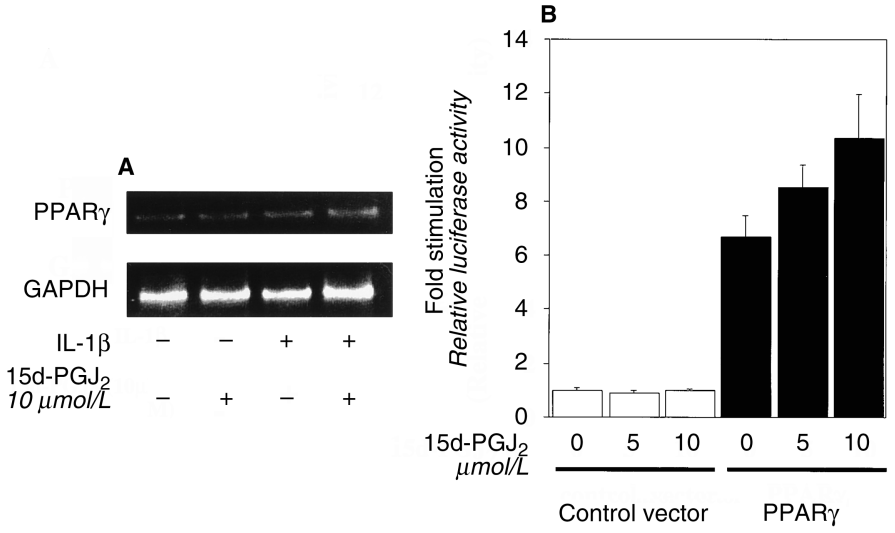

Fig. 4. 15d-PGJ$_2$ does not affect PPARγ mRNA expression and PPARγ-dependent transcription. (*A*) Cells were treated with IL-1β for 4 hours in the presence or absence of 10 μmol/L 15d-PGJ$_2$. PPARγ and GAPDH mRNA expression were assessed by RT-PCR of total RNA prepared from mesangial cells. A representative one of five similar results is shown. (*B*) Mesangial cells were co-transfected with PPRE-luc plasmid and either control vector or PPARγ expression vector, and then treated with 15d-PGJ$_2$ or control buffer for 24 hours. Luciferase activity was measured and normalized against β-galactosidase activity. The results are expressed as relative light units. The results are mean ± SD of five independent experiments.

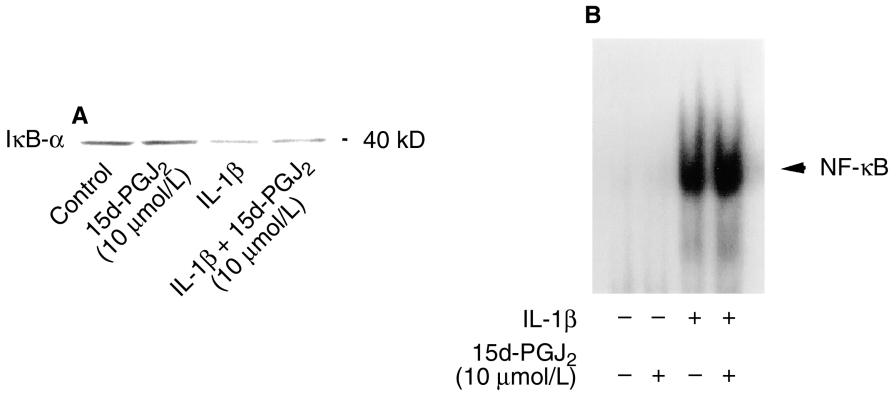

Fig. 5. 15D-PGJ$_2$ does not inhibit the degradation of IκBα and the DNA binding activity of nuclear factor-κB (NF-κB) by IL-1β. The cells were treated with 10 μmol/L 15d-PGJ$_2$ for 30 minutes and stimulated with 1000 pg/mL IL-1β for 60 minutes. (*A*) Whole cell lysates of rat mesangial cells were subjected to immunoblot analysis with anti-IκBα antibody. (*B*) Nuclear extracts were prepared from the cells and incubated with ^{32}P-end labeled NF-κB consensus oligonucleotide probe and subjected to a 4% polyacrylamide gel. The arrow shows the specific binding of NF-κB. The results are representative of five independent experiments that yielded similar results.

[39]. In resting cells, NF-κB is localized in the cytoplasm by association with the inhibitory protein IκB. In response to inflammatory cytokine signals, IκB kinase (IKK) is activated and phosphorylates IκB. Then, phosphorylated IκB is degraded, and free NF-κB migrates into the nucleus and activates target gene expression [40]. Recent studies have indicated that 15d-PGJ$_2$ inhibits IKK activity and DNA binding activity of NF-κB by a PPARγ-independent mechanism [29, 30], and suppresses LPS-stimulated COX-2 expression in macrophages [28]. Thus, we examined the effect of 15d-PGJ$_2$ on NF-κB activation in mesangial cells. IL-1β induced IκBα degradation and an increase in DNA binding activity of NF-κB complex at 60 minutes. 15d-PGJ$_2$ was unable to inhibit either the IL-1β–induced IκBα degradation (Fig. 5A) or the enhancement of DNA binding activity of NF-κB (Fig. 5B).

Effect of 15d-PGJ$_2$ on IL-1β–induced phosphorylation of MAPKs

Interleukin-1β also was shown to activate MAPKs and these kinases were found to play a role in COX-2 expression [41–44]. Thus, we next examined the effect of 15d-PGJ$_2$ on MAPK cascade activated by IL-1β. To determine the role of ERK in IL-1β–induced COX-2 expression, we first examined the effects of PD 98059 and U 0126, inhibitors of MAPK or ERK kinase (MEK), on COX-2 protein production. As shown in Figure 6A, treating the cells with 20 μmol/L PD 98059 or U 0126 inhibited IL-1β–induced COX-2 protein synthesis. Thus, the ERK pathway is considered to be involved in IL-1β–induced COX-2 expression. The phosphorylation of both p44 and p42 ERKs (ERK1 and ERK2) was enhanced by IL-1β and the treatment with 15d-PGJ$_2$ resulted in a significant reduction in IL-1β-induced phosphorylation of ERK (Fig. 6B). We also examined the effect of 15d-PGJ$_2$ on JNK and p38 MAPK pathways and found that 15d-PGJ$_2$ was able to prevent the phosphorylation of JNK by IL-1β (Fig. 6C), but the phosphorylation of p38 MAPK was not inhibited by the treatment of 15d-PGJ$_2$ (Fig. 6D).

To determine whether de novo protein synthesis was required in the inhibitory function of 15d-PGJ$_2$ on ERK

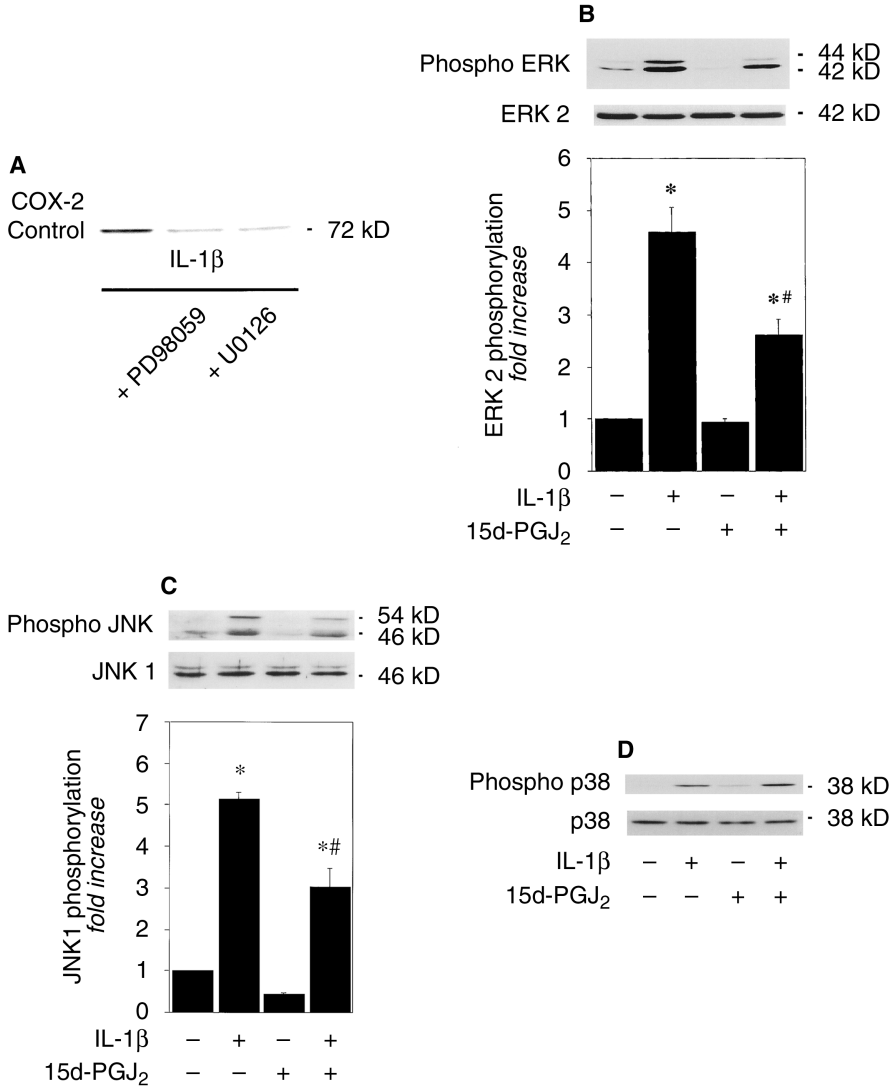

Fig. 6. 15D-PGJ$_2$ suppresses IL-1β–induced activation of mitogen-activated protein kinases (MAPKs). (*A*) The cells were treated with 20 μmol/L PD 98059 or U 0126 for 90 minutes, and stimulated with 1000 pg/mL IL-1β for 8 hours. COX-2 protein production was evaluated by immunoblot analysis with anti-COX-2 antibody. A representative one of five similar results is shown. (*B*) The cells were incubated in the presence or absence 10 μmol/L 15d-PGJ$_2$ for 30 minutes and stimulated with 1000 pg/mL IL-1β for 15 minutes. The samples were electrophoresed on 12% SDS-PAGE. The activities of ERK were evaluated by measuring the phosphorylation by immunoblot analysis with anti-phospho ERK antibody. After stripping, the membranes were reprobed with anti-ERK2 antibody. A representative one of five independent experiments that yielded similar results is shown in the upper panel. Densitometric quantification of phosphorylated ERK2 was performed using NIH image software, and the ratio of phosphorylated ERK2 to total ERK2 is shown in the lower panel. The data are expressed as mean ± SD ($N = 5$). *$P < 0.01$ vs. control, #$P < 0.01$ vs. IL-1β. (*C*) The activities of JNK were evaluated with anti-phospho JNK antibody and anti-JNK1 antibody. A representative one of five results is shown in the upper panel and the ratio of phosphorylated JNK1 to total JNK1 is shown in the lower panel. The data are expressed as mean ± SD ($N = 5$). *$P < 0.01$ vs. control, #$P < 0.05$ vs. IL-1β. (*D*) The activities of p38 MAPK were evaluated with anti-phospho p38 MAPK antibody and anti-p38 MAPK antibody. A representative one of five results is shown.

and JNK pathway, the effects of cyclohexamide were examined. 15d-PGJ$_2$ could suppress the phosphorylation of these kinases even in the presence of 10 μg/mL cyclohexamide (Fig. 7), indicating that the 15d-PGJ$_2$–induced down-regulation of ERK and JNK does not require de novo protein synthesis.

Effect of 15d-PGJ$_2$ on DNA binding activities of AP-1

Activated ERK and JNK enhance c-Fos expression and c-Jun phosphorylation, respectively, and they are found to form AP-1 heterodimers and enhance the DNA binding activity [45–47]. Next, we examined the effect of 15d-PGJ$_2$ on its binding by using consensus AP-1 oligonucleotide. As shown in Figure 8A, IL-1β increased DNA binding activity of AP-1 complex at 60 minutes, and pre-treatment of the cells with 15d-PGJ$_2$ partially inhibited the IL-1β–induced activation of DNA binding activity of AP-1 in EMSA.

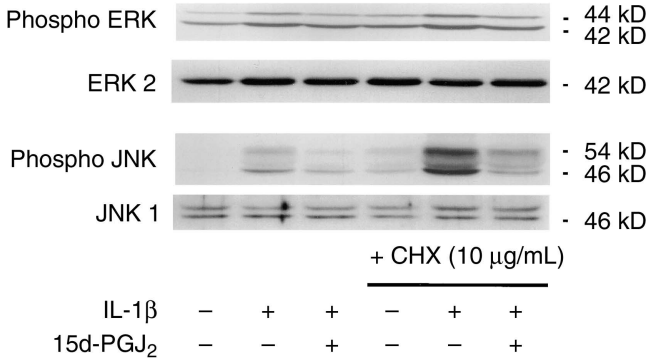

Fig. 7. De novo protein synthesis is not required for the inhibitory effect of 15d-PGJ$_2$ on IL-1β–induced activation of ERK and JNK. The cells were pretreated with 10 μg/mL cyclohexamide (CHX) for 2 hours and incubated in the presence or absence of 10 μmol/L 15d-PGJ$_2$ for 30 minutes. Afterwards, the cells were stimulated with 1000 pg/mL IL-1β for 15 minutes. The activities of ERK and JNK were evaluated by immunoblot analysis. A representative one of five similar results is shown.

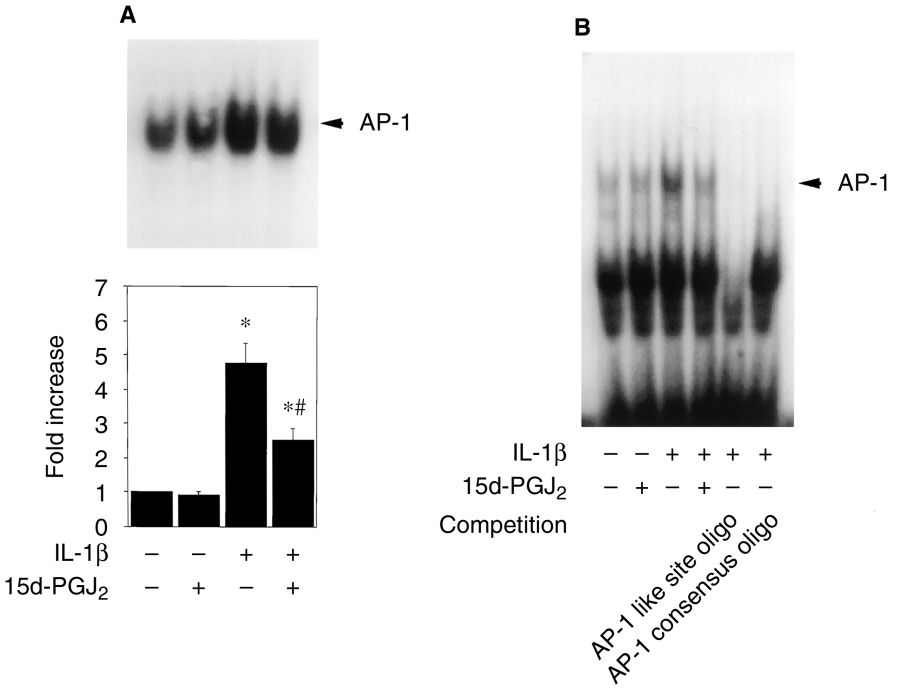

Fig. 8. 15d-PGJ$_2$ inhibits DNA binding activities of activator protein-1 (AP-1). The cells were treated with 10 μmol/L 15d-PGJ$_2$ for 30 minutes and stimulated with 1000 pg/mL IL-1β for 60 minutes. (*A*) Nuclear extracts prepared from the cells were incubated with ^{32}P-end labeled AP-1 consensus oligonucleotide probe, and then subjected to a 4% polyacrylamide gel. The arrow shows the specific binding of AP-1. A representative one of five results is shown in the upper panel. The results of a densitometric analysis of five independent experiments are shown in the lower panel. $*P <$ 0.01 vs. control, $\#P < 0.01$ vs. IL-1β. (*B*) Nuclear extracts were incubated with ^{32}P-end labeled COX-2 promoter oligonucleotide probe containing AP-1–like site. A representative one of five similar results is shown.

To confirm the AP-1 DNA binding in COX-2 promoter, we next used 27 bp double-stranded oligonucleotide (−173/−147) in rat COX-2 promoter containing the AP-1–like sequence. Similarly to the results of Figure 8A, IL-1β enhanced the binding activities of nuclear proteins to this AP-1–like site of COX-2 promoter, and this binding was inhibited by 15d-PGJ$_2$ (Fig. 8B). The specific band was disappeared by the addition of excess amount of either unlabeled AP-1 like oligonucleotide or consensus AP-1 oligonucleotide.

Effect of 15d-PGJ$_2$ on COX-2 promoter activity

To define the effect of 15d-PGJ$_2$ on the COX-2 transcription via the AP-1–like site, we transiently transfected the cells with the rat COX-2 promoter construct (−373/+24) containing the AP-1–like site and examined rat COX-2 promoter activity. As shown in Figure 9, IL-1β stimulated COX-2 promoter activity by 2.2-fold, and this effect was partially inhibited by 15d-PGJ$_2$, which did not affect the basal COX-2 promoter activity. However, when the mutated COX-2 promoter construct was transfected, the basal activity of COX-2 promoter activity was reduced and IL-1β failed to stimulate this promoter activity in mesangial cells (Fig. 9).

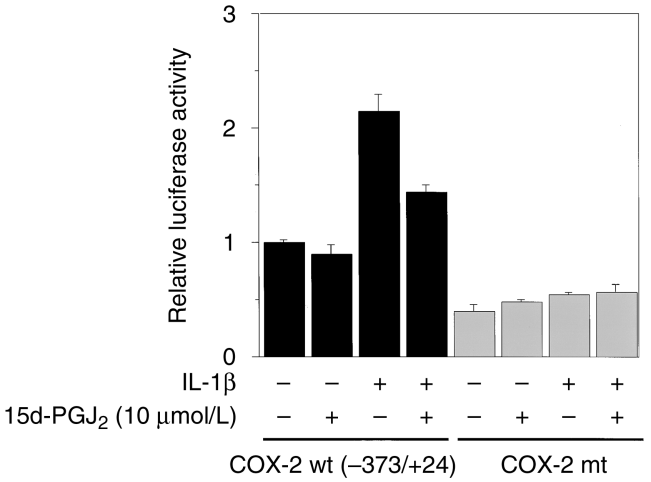

Fig. 9. 15d-PGJ$_2$ inhibits IL-1β−induced COX-2 promoter activity. Mesangial cells transfected with either 0.8 μg of the rat COX-2 promoter (−373/+24) reporter plasmid (wt) or COX2-AP-1 like site mutant reporter plasmid (mt) were treated with 10 μmol/L 15d-PGJ$_2$ for 30 minutes and then incubated with or without 1000 pg/mL IL-1β for 24 hours. Luciferase activity was measured and normalized against β-galactosidase activity. Results are expressed as relative light units. The results are mean ± SD of five independent experiments.

DISCUSSION

This study clearly demonstrates that 15d-PGJ$_2$ suppresses the IL-1β–induced increase in the expression of mRNA and protein of COX-2 in glomerular mesangial cells. IL-1β–induced production of PGE$_2$, one of the main prostaglandins catalyzed by COX-2 in the kidney, also was inhibited by 15d-PGJ$_2$. Furthermore, we found that 15d-PGJ$_2$ inhibited IL-1β–induced activation of ERK, JNK, and AP-1, which could regulate COX-2 gene expression.

Cyclooxygenase-2, an inducible form of cyclooxygenase, is a key enzyme in prostaglandin synthesis and is

known to play an important role in the process of glomerular injury [48]. Thus, it is important to understand the mechanism of the regulation of COX-2 expression in glomerular mesangial cells, as these cells play a major role in the pathological process of glomerular inflammation. One of the primary products of COX is prostaglandin D_2 (PGD_2), which is rapidly and spontaneously converted to PGJ_2, $\Delta^{12}PGJ_2$, and finally 15d-PGJ_2 by non-enzymatic dehydration. J-series prostaglandins have unique biological effects, such as the suppression of viral replication and inflammation [49]. Moreover, 15d-PGJ_2 is known to be one of the natural ligands of PPARγ [20, 21] and PPARγ ligands were shown to have anti-inflammatory actions in various cells [23, 24, 50, 51]. Recent studies have reported that 15d-PGJ_2 inhibits COX-2 expression via PPARγ-dependent manner in human epithelial cells [52] and in macrophages [28], suggesting that 15d-PGJ_2 works as an intracellular mediator in negative feedback loop of COX-2 through PPARγ [28].

We first hypothesized that 15d-PGJ_2 might have anti-inflammatory effects through the activation of PPARγ in mesangial cells because several reports demonstrated that PPARγ was expressed in mesangial cells [53–58], and PPARγ agonists were shown to modulate mesangial cell proliferation and differentiation [53]. We showed the presence of PPARγ mRNA in rat mesangial cells by RT-PCR method, but not by Northern blot analysis (data not shown). We also demonstrated that 15d-PGJ_2 or IL-1β did not affect PPARγ mRNA expression. Although our results clearly indicated that 15d-PGJ_2 was able to inhibit the IL-1β–induced increase in the expression of mRNA and protein of COX-2 in mesangial cells, PPARγ synthetic agonists, troglitazone and pioglitazone, did not inhibit COX-2 expression. Furthermore, 15d-PGJ_2 could up-regulate PPRE luciferase activity in a dose-dependent manner only in PPARγ-overexpressed mesangial cells, and not in the cells transfected with control vector. Thus, the action of 15d-PGJ_2 on COX-2 expression and PGE2 production seems to be independent of its ability to activate PPARγ in mesangial cells.

A possible PPARγ-independent mechanism shown in other cell types is the inhibition of IKK activity and DNA binding activity of nuclear NF-κB by 15d-PGJ_2 [29, 30]. A recent study indicated that the treatment of human mesangial cells with 15d-PGJ_2 blocked IL-1β–induced MCP-1 mRNA expression and protein production by inhibiting the NF-κB pathway [58]. However, in our study, 15d-PGJ_2 was not able to inhibit IL-1β–induced IκBα degradation and increase in DNA binding activity of NF-κB, suggesting that the NF-κB pathways was not involved in the inhibitory effect of 15d-PGJ_2 on the COX2 expression in rat mesangial cells.

The MAPK family of serine/threonine protein kinases is a vital signaling mechanism that transmits signals from the cell surface to the nucleus to ultimately regulate gene expression [59] and plays an important role in the signaling of various stimuli in mesangial cells [60–62]. We and others have reported that IL-1β is able to activate ERK, JNK, and p38 MAPK [42–44, 62]. A previous study suggested that the activation of both JNK and p38 is required for IL-1β–induced COX-2 expression [44]. Furthermore, by using the specific inhibitors of MEK, we demonstrated that the activation of ERK pathway is an important mechanism to mediate COX-2 expression by IL-1β. Recent studies have indicated that 15d-PGJ_2 reduces IL-1–induced JNK phosphorylation in a rat insulinoma cell line [51] and LPS-induced ERK phosphorylation in macrophages [63]. Since our study found that 15d-PGJ_2 was able to inhibit IL-1β–induced activation of ERK and JNK pathways, the inhibition of these kinases is one of the possible mechanisms of 15d-PGJ_2.

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 and other PPARγ ligands have been reported to induce the heat shock proteins (HSPs) and heme oxygenase-1 (HO-1) [22, 50], and the induction of these proteins has been proposed to be associated with the inhibitory effect of 15d-PGJ_2 on signaling events. In rat islets, 15d-PGJ_2 suppresses IL-1–induced JNK activation by inducing heat shock protein 70 [51]. In our study, 15d-PGJ_2 was able to inhibit IL-1β–induced activation of ERK and JNK even in the presence of cyclohexamide. Thus, de novo protein synthesis including HSPs and HO-1 seems not to be required for the inhibitory effect of 15d-PGJ_2 on ERK and JNK pathways.

Because IL-1β stimulated the DNA binding activity of AP-1 [59, 62], we examined the effect of 15d-PGJ_2 on the DNA binding activity of AP-1. We found that the IL-1β–induced increase in DNA binding activity of AP-1 was inhibited by 15d-PGJ_2 in mesangial cells. We also demonstrated that both basal and IL-1β–stimulated COX-2 promoter activities were reduced when the AP-1–like site was mutated. Thus, these results implicate that 15d-PGJ_2 inhibits IL-1β–induced COX-2 expression by an anti–AP-1 mechanism, providing an important PPARγ-independent mechanism of 15d-PGJ_2's action.

A recent study reported that PPARγ ligands can reduce phorbol ester-induced COX-2 by suppressing the AP-1 activity, and one consequence of this anti–AP-1 effect is a competition for limiting the amounts of general coactivator CBP/p300 [52]. Since the precise mechanism of the action of PPARγ ligands is not clarified yet, further study is necessary to elucidate the precise mechanism of the inhibitory effect of 15d-PGJ_2 on MAPKs and AP-1 activation in mesangial cells.

The selective COX-2 inhibitor, flosulide, has been reported to decrease proteinuria in rats with Heymann nephritis [64, 65]. Furthermore, another COX-2 inhibitor, SC 58236, has been shown to decrease proteinuria and inhibit the development of glomerular sclerosis in rats with reduced kidney mass [66]. These data suggest that the suppression of COX-2 might be useful for treat-

ment of glomerulonephritis. Here, we present evidence that 15d-PGJ$_2$ is able to inhibit IL-1β–induced COX-2 expression and PGE$_2$ production. Thus, 15d-PGJ$_2$ might be expected to have similar renoprotective effects as the COX-2 selective inhibitors.

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