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15-Deoxy-$\Delta^{12,14}$-Prostaglandin-J2 and Ciglitazone Inhibit TNF-α-Induced Matrix Metalloproteinase 13 Production Via the Antagonism of NF-κB Activation in Human Synovial Fibroblasts

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Collagenase-3 (matrix metalloproteinase, MMP-13) plays an important role in the degradation of cartilage in pathologic conditions. MMP-13 is elevated in joint tissues in both rheumatoid arthritis (RA) and osteoarthritis (OA). In addition, inflammation-stimulated synovial fibroblasts are able to release MMP-13 and other cytokines in these diseases. The peroxisome proliferator-activated receptor-γ (PPARγ) ligands are recently considered as new anti-inflammatory compounds and these ligands were reported to ameliorate inflammatory arthritis. The aim of this study is to evaluate the mechanisms how PPARγ ligands inhibit the inflammatory response in synovial fibroblasts. Two PPARγ ligands, cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin-J2 (15d-PGJ2) and synthetic thiazolidinedione compound ciglitazone were examined in this study. Here we found that 15d-PGJ2 and ciglitazone markedly inhibited TNF-α-induced MMP-13 production in human synovial fibroblasts. In addition, activation of nuclear factor κB (NF-κB) is strongly associated with MMP-13 induction by TNF-α and the activation of NF-κB was determined by Western blot, reporter assay, and immunofluorescence. It was found that 15d-PGJ2 markedly attenuated the translocation of NF-κB by direct inhibition of the activation of IKK via a PPARγ-independent manner. Ciglitazone also inhibits TNF-α-induced MMP-13 expression by suppressing NF-κB activation mainly via the modulation of p38-MAPK. Collectively, our data demonstrate that 15d-PGJ2 and ciglitazone attenuated TNF-α-induced MMP-13 expression in synovial fibroblasts primarily through the modulation of NF-κB signaling pathways. These compounds may have therapeutic application in inflammatory arthritis.

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Rheumatoid arthritis (RA) is a chronic and systemic autoimmune syndrome, which is characterized by massive synovial proliferation and inflammation and leads to the destruction of joint cartilage (Brennan and McInnes, 2008). Osteoarthritis (OA) is a group of joint degenerative disease, which is induced by joint injury, obesity, and aging (Poole, 1999). However, in a great part of OA patients, onset is spontaneous and not directly related to trauma or weight-bearing sites. Recent reports indicate that synovial inflammation is implicated in many of the signs and symptoms of OA (Pelletier et al., 2001; Bondeson et al., 2007). Taken together, these two diseases constitute a major part of arthritis and the common feature of inflammatory synovium is related to the degeneration of articular cartilage. There is an increase of pro-inflammatory cytokines including tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) produced by inflammatory cells, synovial fibroblasts, and chondrocytes (Goldring, 2000; Brennan and McInnes, 2008). Cartilage extracellular matrix (ECM) contributed by chondrocytes provides the mechanical strength and flexibility to joints. However, cartilage ECM is the target of matrix metalloproteinases (MMPs), especially MMP-13 (collagenase-3) (Yasuda, 2006). MMP-13 released by synovial fibroblasts and chondrocytes is able to cleave type II collagen derived from cartilage (Knauper et al., 1996) and plays a pivotal role in the destruction of cartilage in RA and OA (Kanbe et al., 2004; Yasuda, 2006; Su et al., 2009).

Supporting information may be found in the online version of this article.

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In arthritic joints, the synovial fibroblast is the main target of inflammation and acts together with chondrocytes, in response to pro-inflammatory cytokines such as TNF-α and IL-1β, which are mainly produced by infiltrated inflammatory cells including macrophages, synovial fibroblasts, to produce chemokines which promote inflammation, angiogenesis, and cartilage destruction via matrix-degrading enzymes including MMP-13 (Sun and Yokota, 2002; Brennan and McInnes, 2008).

Peroxisome proliferator-activated receptors gamma (PPARγ) is a member of ligand-activated transcription factor, which functions as a heterodimer with a retinoid X receptor and binds to the PPAR responsive element (PPRE) within the promoter of the target genes (Kersten et al., 2000; Rosen and Spiegelman, 2001). PPARγ can be activated by a wide variety of substances such as long chain fatty acids, cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin-J2 (15d-PGJ2), and thiazolidinediones (TZDs). PPARγ was initially considered as a pivotal regulator of lipid metabolism and homeostasis (Kersten et al., 2000). In addition, accumulating data suggest a possible role for PPARγ ligands in suppressing inflammatory response. It has been reported that 15d-PGJ2 and TZD compounds exert potent anti-inflammatory effects on immune response cells including macrophages, microglia, astrocytes, neutrophils, and lymphocytes (Asada et al., 2004; Buckingham 2005; Consoli and Devangelio 2005; Storer et al., 2005; Phulwani et al., 2006; Hounoki et al., 2008).

Cyclopentenone prostaglandin 15d-PGJ2, the endogenous PPARγ ligand, is a COX-2-derived prostaglandin and regulates human autoimmune diseases including inflammatory arthritis. 15d-PGJ2 is a downstream metabolite of PGD2 and its synthesis initially depends upon the dehydration of PGD2 (Li et al., 2001; Scher and Pillinger, 2005). 15d-PGJ2 can be produced in a variety of cells, which display high COX-2 activity, including mast cells, T cells, Kupffer cells, hepatic myofibroblasts, and alveolar macrophages (Li et al., 2001; Kim et al., 2007). 15d-PGJ2 exerts anti-inflammatory action in arthritis and inhibits LPS- or IL-1β-induced inflammatory response in synovial fibroblasts (Simonin et al., 2002; Farrajota et al., 2005). On the other hand, the TZD compounds such as rosiglitazone (Avandia) and pioglitazone (Actos) are common oral insulin-sensitizing anti-diabetic agents mediated by their interaction with PPARγ. However, they are reported to inhibit inflammatory response in brain system (Ji et al., 2010; Morgenweck et al., 2010). The other TZD compound ciglitazone is also reported to attenuate inflammation response in monocytes (Syrovets et al., 2002) and in astrocytes (Phulwani et al., 2006) and another TZD compound troglitazone is reported to inhibit endogenous production of cytokines and reduce the DNA binding activity of NF-κB in response to TNF-α or IL-1β (Yamasaki et al., 2002). Pioglitazone and troglitazone are also reported to ameliorate the symptoms of arthritis (Kawahito et al., 2000; Bongartz et al., 2005). However, some anti-inflammatory effects of 15d-PGJ2 and ciglitazone are likely to be PPARγ-independent (Chawla et al., 2001; Phulwani et al., 2006). At least two identified candidates are reported to mediate PPARγ-independent actions of 15d-PGJ2, the NF-κB systems and the extracellular signal-related kinase (ERK) signaling pathways (Scher and Pillinger, 2005). Although 15d-PGJ2 and TZD compounds are reported to be important anti-inflammatory mediators and may be useful in the treatment of RA (Kawahito et al., 2000), the mechanism regarding how 15d-PGJ2 and TZD compounds inhibit the inflammatory response including destructive collagenase MMP-13 expression in synovial fibroblasts is still unclear.

In this study, we examined the effects of 15d-PGJ2 and ciglitazone on TNF-α-induced MMP-13 release in human synovial fibroblasts. It was found that both 15d-PGJ2 and ciglitazone significantly inhibited TNF-α-induced MMP-13 expression through a PPARγ-independent pathway and acted via the direct inhibition of NF-κB signaling.

Materials and Methods

Materials

Mouse monoclonal antibody for α-tubulin, C23, NF-κB p65, and rabbit polyclonal antibody for IgG, IKKα/β, IκBα, NF-κB p50, NF-κB p65, and goat anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody for phosphor-p38-MAPK or phosphor-IκBα and rabbit monoclonal antibody for phosphor-IKKα/β were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody for human CD90 was purchased from BD Bioscience (San Jose, CA). The p38 dominant negative mutant was provided by Dr. J. Han (South-western Medical Center, Dallas, TX). 15d-PGJ2 was from Cayman Chemical Company (Ann Arbor, MI). Ciglitazone, collagenase, 4′,6-diamidino-2-phenylindole (DAPI), pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma–Aldrich (St. Louis, MO). Recombinant human TNF-α, and enhanced chemiluminescent HRP substrate (ECL) were from Millipore (Bedford, MA). MMLV Reverse Transcriptase and reporter assay kit were purchased from Promega (Madison, WI). Taqman PCR Master Mix and qPCR probes were purchased from Applied Biosystems (Foster City, CA). We purchased RPMI-1640 medium, trypsin and anti-rabbit secondary antibody conjugated with Alexa Fluor 488 from Invitrogen (Carlsbad, CA) and fetal bovine serum (FBS) from Biological Industries (Kibbutz Beit Haemek, Israel), 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662) from Tocris Bioscience (Ellisville, MO), tri-zol and tag DNA polymerase from MDBIO (Taipei, Taiwan). PCR primers were from GeneMark (Taichung, Taiwan).

Cell cultures

Human synovial fibroblasts were isolated by collagenase treatment from synovial tissues obtained from patients with RA undergoing total knee replacement surgeries (Tang et al., 2007) after approval of hospital's ethics committee (Taichung Veterans General Hospital, Taichung, Taiwan). Patients are fulfilled with diagnostic criteria of American College of Rheumatology (ACR). Fresh synovial tissues were minced and digested in a solution of collagenase and DNase. Isolated synovial fibroblasts were filtered through 70 μm nylon filters. The cells were then grown on culture dishes in 95% air–5% CO_2 with RPMI-1640, which was supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml fungizone (pH 7.6). Over 90% of cultured cells were fibroblasts which were characterized by flow cytometry using CD90 (Thy-1) antibody. Synovial fibroblasts from passages four to nine were used in this study.

RT-PCR for mRNA analysis

mRNA was analyzed by reverse transcription polymerase chain reaction (RT-PCR). Cells were pretreated with test substances and then incubated with TNF-α for 6 h. The supernatant was used for MMP-13 analysis and total RNA was extracted from synovial fibroblasts using TRIzol kit and was quantified by adding 1 μl sample to 79 μl RNase-free water and the absorbance was measured in a RNA/DNA calculator (GeneQuant Pro, GE Healthcare, Piscataway, NJ) at 260 and 280 nm. RNA was used for RT-PCR using two-step MMLV RT kit. Amplification by Tag DNA polymerase was accomplished at 30–35 cycles, which was within the linear change of PCR products (Lin et al., 2007). In brief, sense and anti-sense primers were added and the following profile was used: 1 cycle for 94°C for 3 min, followed by set cycles at 94°C for 45 sec; 55°C for 45 sec and 72°C for 45 sec. All primers used are listed as follows:

MMP-13, sense: TTGAGGATACAGGCAAGACT,
anti-sense: TGGAAGTATTACCCCAATG, 33–35 cycles;
GAPDH, sense, GCCATCAACGCCCCTTCATTGAC;
anti-sense, ACGGAAGGCCATGCCAGTGAGCTT, 30 cycles.

PCR products were then separated electrophoretically using a 2% agarose DNA gel stained with ethidium bromide. The mRNA levels were normalized to the levels of GAPDH.

Quantitative Real-Time PCR for mRNA analysis

The RNA was obtained as described above and 1 μg total RNA was used for RT-PCR using MMLV RT kit. The expression of human MMP-13 was measured by quantitative PCR using specific probe (Taqman gene expression assay: Hs00233992_m1) and Taqman PCR Master Mix. Amplification was performed in the following cycling conditions: 95°C for 10 min and then 40 cycles of 95°C for 15 sec followed by 60°C for 1 min (Lin et al., 2010). The optimal concentrations of probes and templates that were used in each reaction were established based on the standard curve created before the reaction and corresponded to the nearly 100% efficiency of the reaction. The reference household gene used to normalize the amount of mRNA in the cultures was human GAPDH (HS9999905_m1). The fold change in gene expression relative to the control was calculated by $2^{-\Delta\Delta CT}$.

Measurement of MMP-13 production

Synovial fibroblasts were cultured in six-well plates until confluent. The medium was replaced with serum-free medium and test substances were then added. The level of MMP-13 in conditioned medium from synovial fibroblasts was measured by a specific immunoassay, Quantikine (R & D System, Minneapolis, MN), according to the procedure described by the manufacturer (Zayed et al., 2008). Briefly, 100 μl of assay diluent and 50 μl of culture supernatant were loaded into per well which is pre-coated with the monoclonal antibody which is specific for MMP-13 for 2 h. After four times of washes, 200 μl of enzyme-linked monoclonal antibody for MMP-13 was added into each well for another 2 h. After washes, 200 μl of substrate solution was added to each well to develop in the dark for 30 min and 50 μl of stop solution was then added to stop the reaction. The concentrations of MMP-13 were determined using MMP-13 standard. The absorbance at 450 nm was determined using microplate reader (Bio-Tek, Winooski, VT).

Transfection and reporter gene assay

The κB-luciferase activity was measured after transfection with a reporter plasmid which contains the NF-κB binding site in the promoter region of the reporter luciferase gene (Lu et al., 2007). The cotransfection with κB-luciferase plasmid and β-galactosidase expression vector was performed using the Amaxa Nucleofector II (Amaxa, Cologne, Germany) (Shin et al., 2010). Two micrograms of κB-luciferase plasmid and 1 μg β-galactosidase expression vector were mixed with 0.1 ml cell suspension containing 8×10^5 synovial fibroblasts and transferred to an electroporation cuvette and then transfected by electroporation using the A24 pulsing program. After electroporation, transfected synovial fibroblasts were immediately transferred to six-well plates containing 2 ml of growth medium for 24 h. The medium was replaced with serum-free medium and incubated with test substances for 30 min and TNF-α was then added for 6 h. After washes, 100 μl of reporter lysis buffer (Promega) was added and the cells were scraped from six-well plates. The supernatant was collected after centrifugation at 12,000*g* at 4°C for 2 min. Aliquots of 20 μl of cell lysates containing equal amounts of protein (20–30 μg) were added into wells of an opaque black 96-well plate and 100 μl of luciferase substract was then added to each sample. The luminescence was measured in an Orion II microplate luminometer (Berthold, Pforzheim, Germany). The luciferase activity value was normalized to the activity of cotransfected β-galactosidase.

Transfection of dominant negative mutant

The p38 dominant negative mutant (Chiu et al., 2008) or control plasmid (pLKO.1, National RNAi Core, Taipei, Taiwan) was transfected into synovial fibroblasts using Amaxa Nucleofector II. Briefly, 8×10^5 synovial fibroblasts were transfected with 3 μg p38 dominant negative mutant or control plasmid. Cells were transfected by electroporation using A24 pulsing program (Shin et al., 2010). After 24 h, the growth medium was replaced with serum-free medium and test substances.

Western Blot

Synovial fibroblasts were seeded onto six-well plates. After reaching confluence, cells were incubated with test substances, and then washed with cold PBS and lysed for 30 min at 4°C with lysis buffer as described previously (Lin et al., 2010). For the separation of cytoplasmic extracts (CE) and nuclear extracts, cells were cultured onto 10 cm dish. After reaching confluence, cells were treated with test substances, CE, and nuclear extracts were separated by NE-PER (Thermo Scientific-Pierce, Rockford, IL). Equal protein (30 μg) was applied per lane, and electrophoresis was performed under denaturing conditions on a 10% SDS gel and transferred to an immobilon-P (PVDF) membrane (Millipore). The blots were blocked with 5% nonfat milk in TBS-T (0.5% Tween 20 in 20 mM Tris and 137 mM NaCl) for 1 h at room temperature and then probed with antibodies against phosphor-p38-MAPK, phosphor-IκBα, IκBα, phosphor-IKKα/β, IKKα/β, NF-κB p50, NF-κB p65 (1:1,000) at 4°C overnight. After three washes by TBS-T, the blots were subsequently incubated with goat anti-rabbit or anti-mouse peroxidase-conjugated secondary antibody (1:10,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Amersham Hyperfilm™ ECL (GE Healthcare, Pollards Wood, UK) or Biospectrum Imaging System (UVP, Upland, CA). For normalization purposes, the same blot was also probed with mouse anti-α-tubulin antibody or mouse anti-C23 antibody (1:1,000).

Immunofluorescent staining

For immunolabeling studies, synovial fibroblasts were seeded on glass overnight and then treated with test substances. Cells were fixed by 1% paraformaldehyde for 15 min and incubated in 4% BSA for nonspecific blocking. Synovial fibroblasts were stained with primary mouse monoclonal antibody against NF-κB-p65 (SC-8008, 1:200) (Biswas et al., 2004; Korcok et al., 2005) and then with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen). The nucleus was stained by DAPI and the confocal images were obtained using excitation wavelength 494 nm and emission wavelength 519 nm, respectively (for Alexa Fluor 488) (model SP5 TCS; Leica, Heidelberg, Germany).

Statistics

The data given are mean ± SEM. The significance of difference between the experimental group and control was assessed by one-way analysis of variance (ANOVA) and 2-tailed Student's *t*-test. The difference is significant if the *P* value is less than 0.05.

Results

15d-PGJ2 and ciglitazone inhibit TNF-α-induced MMP-13 expression in synovial fibroblast

TNF-α is a potent inflammatory cytokine and is involved in cartilage degradation. Treatment of TNF-α (10 ng/ml) for 6 h markedly increased the mRNA expression and protein production of MMP-13 in synovial fibroblasts about three to fourfold, respectively (Fig. 1). Pretreatment of synovial

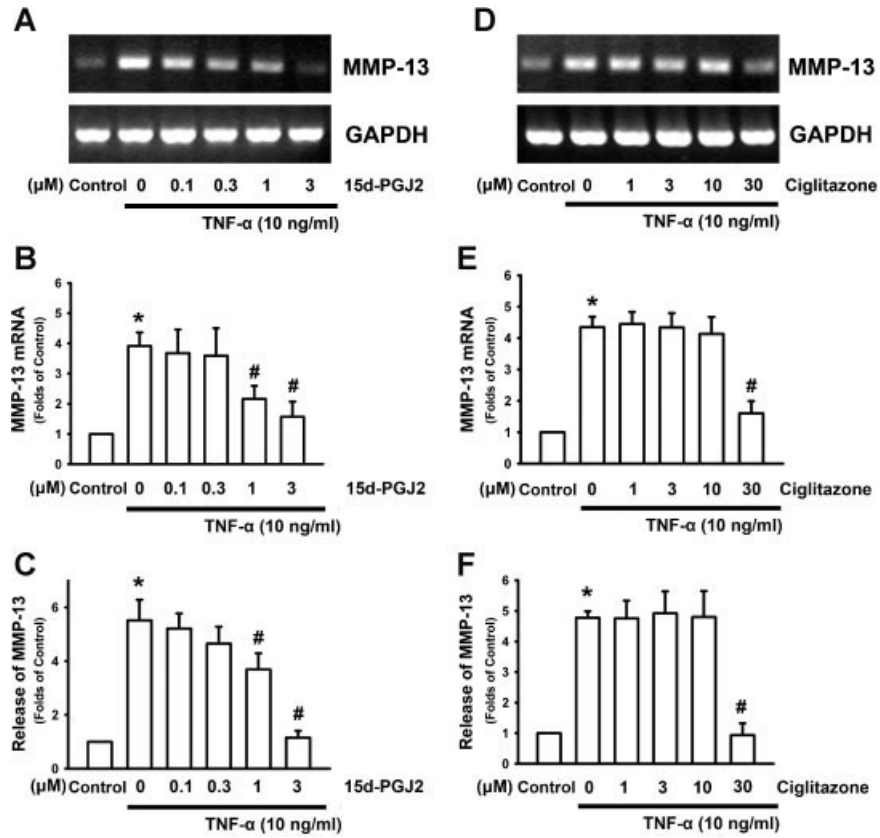

Fig. 1. Inhibition of TNF-α-induced MMP-13 expression by 15d-PGJ2 and ciglitazone. Synovial fibroblasts were pretreated with various concentrations of ciglitazone and 15d-PGJ2 for 30 min and then treated with TNF-α (10 ng/ml) for 6 h, mRNA was extracted by Tri-Zol kit and the culture medium was collected for released MMP-13 analysis. RT-PCR showed that TNF-α significantly increased mRNA levels of MMP-13. Pretreatment of 15d-PGJ2 (1–3 μM) or ciglitazone (30 μM) inhibited the TNF-α-induced MMP-13 mRNA expression (A,D) (n = 6), the quantitative data were shown in (B) and (E), respectively. In addition, TNF-α increased the production and release of MMP-13. 15d-PGJ2 (C) and Ciglitazone (30 μM) (F) significantly abrogated the MMP-13 induction by TNF-α (n = 4). Data are presented as mean ± SEM. *$P < 0.05$ as compared with control group. #$P < 0.05$ as compared with TNF-α stimulation alone.

fibroblasts with 15d-PGJ2 (0.1–3 μM) significantly inhibited TNF-α-induced MMP-13 mRNA expression and protein release into supernatant in a concentration-dependent manner (inhibition was up to 81.6 ± 16.1% and 95.5 ± 4.5%, respectively, at 3 μM, Fig. 1A–C). Ciglitazone at a higher concentration of 30 μM also exerted similar inhibitory effects in both mRNA and protein levels (78.1 ± 11.8% and 113.1 ± 5.1%, respectively, Fig. 1D–F). 15d-PGJ2 was much more potent than ciglitazone to inhibit MMP-13 production.

15d-PGJ2 and ciglitzone inhibit TNF-α-induced NF-κB nuclear translocation in a PPARγ-independent manner

NF-κB is implicated in the transcriptional regulation of MMP-13 expression by TNF-α. Bondeson et al. (2007) has demonstrated that the activation of NF-κB plays an important role in the inflammatory action of TNF-α in OA synovial fibroblasts. To examine whether NF-κB is involved in the production of MMP-13 by TNF-α in RA synovial fibroblasts, NF-κB inhibitor PDTC was used in this study. It was found that blockade of NF-κB signaling by PDTC significantly inhibited TNF-α-induced MMP-13 mRNA expression (75.9 ± 19.8, Supplementary Fig. 1A,B) and also protein release into culture medium (45.4 ± 14.4%, Supplementary Fig. 1C).

In order to examine the relationship between NF-κB signaling and 15d-PGJ2 or ciglitazone, we further examined the effect of 15d-PGJ2 and ciglitazone on NF-κB nuclear translocation. As shown in Figure 2A, treatment of TNF-α (10 ng/ml) for 30 min markedly increased the levels of p50 and p65 subunit in nuclear extract when compared to unstimulated cells. Pretreatment of 15d-PGJ2 (3 μM) or ciglitazone (30 μM) inhibited the translocation of NF-κB subunits from cytosol into nucleus. To further examine whether PPARγ signaling is involved in this action or not, a specific potent PPARγ antagonist GW9662 was used (IC50 is at nM range) (Leesnitzer et al., 2002). However, pretreatment of PPARγ antagonist GW9662 even at a higher concentration of 10 μM exerted little effect on the inhibitory action of 15d-PGJ2 or ciglitazone. Immunofluorescent staining also showed that TNF-α markedly increased the translocation of NF-κB from cytosol to nucleus (Fig. 2B). Pretreatment of 15d-PGJ2 or ciglitazone markedly attenuated the nuclear translocation of p65 (Fig. 2B). In addition, the NF-κB activity was measured using κB-luciferase reporter assay. As shown in Figure 3, treatment of TNF-α (10 ng/ml) for 6 h significantly increased κB-luciferase activity and the addition of 15d-PGJ2 (3 μM) or ciglitazone (30 μM) also markedly inhibited TNF-α-induced κB-luciferase activity. These results indicate that inhibition of NF-κB activation is involved in

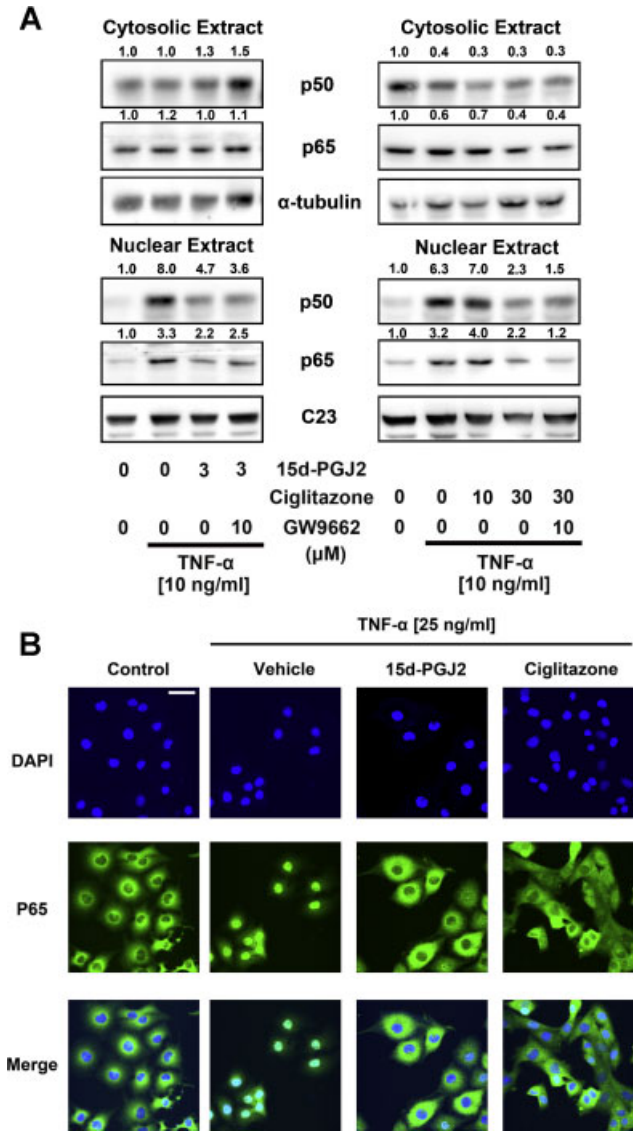

Fig. 2. 15d-PGJ2 and ciglitazone inhibits TNF-α-induced NF-κB translocation in human synovial fibroblasts. A: Cultured synovial fibroblasts were pretreated with ciglitazone or 15d-PGJ2 in the presence of absence of GW9662 (10 μM). TNF-α (10 ng/ml) was then added for another 30 min. Cytosolic and nuclear extracts were separated by NE-PER kit. 15d-PGJ2 (3 μM) and ciglitazone (30 μM) significantly inhibited the nuclear translocation of NF-κB subunits of p50 and p65. PPARγ antagonist GW9662 (10 μM) exerted no influence on this inhibitory effect (n = 4). B: Immunofluorescent staining showed that NF-κB P65 translocated into nucleus after treatment of TNF-α (25 ng/ml) for 30 min and pretreatment of 15d-PGJ2 (3 μM) or ciglitazone (30 μM) antagonized this nuclear translocation (n = 4). Scale bar: 50 μm.

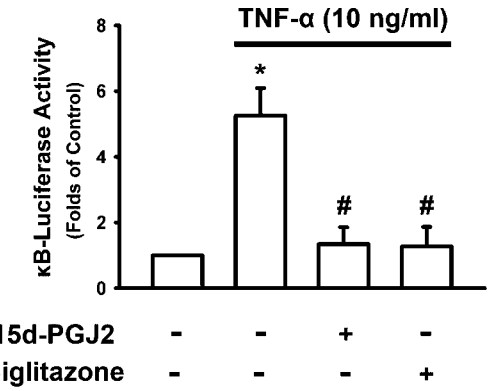

Fig. 3. 15d-PGJ2 and ciglitazone inhibit TNF-α-induced NF-κB reporter activity. Synovial fibroblasts were transfected with κB-luciferase expression vector by electroporation and then pretreated with 15d-PGJ2 (3 μM) or ciglitazone (30 μM) for 30 min before incubation with TNF-α (10 ng/ml) for 6 h. Luciferase activity was then assayed. Note that 15d-PGJ2 and ciglitazone markedly inhibited the κB-luciferase activity (n = 3). Data are presented as mean ± SEM. $^{*}P < 0.05$ as compared with control group. $^{\#}P < 0.05$ as compared with TNF-α stimulation alone.

the antagonism of 15d-PGJ2 or ciglitazone on TNF-α-induced MMP-13 expression.

15d-PGJ2 inhibits TNF-α-induced MMP-13 expression by attenuating IKK and IκBα activation in a PPARγ-independent manner

15-Deoxy-$\Delta^{12,14}$-prostaglandin-J2 was reported to exert strong anti-inflammatory actions in a PPARγ-dependent or -independent manner (Scher and Pillinger, 2005). As mentioned above, 15d-PGJ2 inhibited TNF-α-induced NF-κB translocation in a PPARγ-independent manner. In order to further examine the mechanism of 15d-PGJ2 on the inhibition of TNF-α-induced MMP-13 expression, MMP-13 expression was measured by PCR and ELISA assay and upstream signaling of NF-κB was measured by immunoblotting. It was found that treatment of GW9662 exerted little effects on TNF-α-induced MMP-13, indicating that endogenous PPARγ is not involved in the TNF-α action in synovial fibroblasts (Fig. 4A,B). In addition, pretreatment of GW9662 did not significantly antagonize the inhibitory effects of 15d-PGJ2 on TNF-α-induced MMP-13 mRNA expression and protein production (Fig. 4A–D). The mRNA was evaluated by semi-quantitative (Fig. 4A,B) and real-time quantitative methods (Fig. 4D). These results indicate that 15d-PGJ2 inhibits the induction of MMP-13 mainly through a PPARγ-independent manner. Furthermore, treatment of TNF-α activated IKKα/β (Fig. 4E), which phosphorylates IκBα at Ser32 and Ser36 (DiDonato et al., 1997) to cause the ubiquitination of IκBα at lysine residues and degradation by proteasome. NF-κB can thus translocate from cytosol into nucleus (Thompson et al., 1995). 15d-PGJ2 (3 μM) decreased TNF-α-induced activation of IKKα/β and also the phosphorylation and degradation of IκBα. Pretreatment of PPARγ antagonist GW9662 (3 and 10 μM) did not antagonize these inhibitory actions of 15d-PGJ2. These results indicate that 15d-PGJ2 inhibits TNF-α-induced NF-κB activation by the inhibition of IKK phosphorylation via a PPAR-γ-independent pathway.

Ciglitazone inhibits TNF-α-induced MMP-13 expression by inhibiting both p38-MAPK and NF-κB activation

TZD compound ciglitazone is considered as a high affinity ligand for PPARγ. Ciglitazone is reported to reduce inflammatory response by inhibiting NF-κB activation and DNA binding (Chawla et al., 2001; Syrovets et al., 2002; Consoli and Devangelio, 2005). However, as shown in Figure 5, ciglitazone at 30 μM inhibited TNF-α-induced MMP-13 gene expression (Fig. 5A) and protein release (Fig. 5B), which was not antagonized by GW9662. Therefore, ciglitazone inhibited

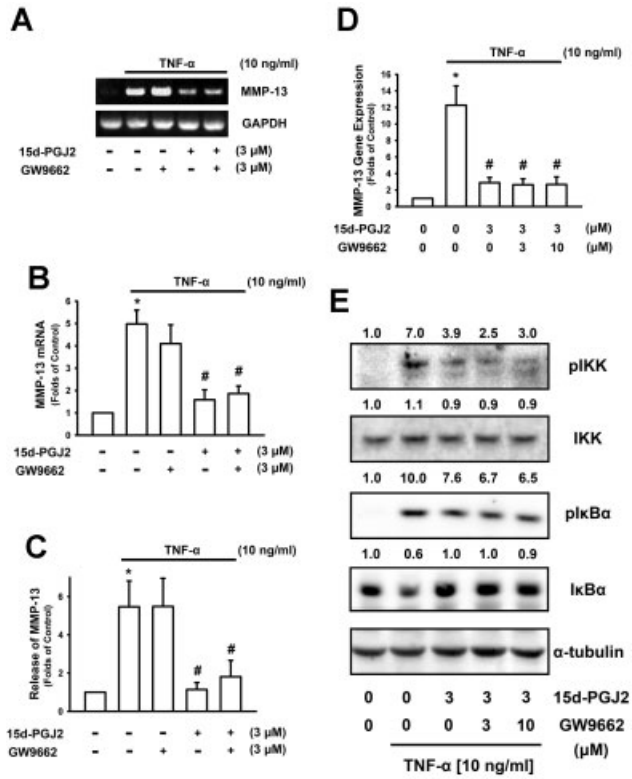

Fig. 4. 15d-PGJ2 inhibits TNF-α-induced MMP-13 expression by reducing IKKα/β activation, IκBα phosphorylation, IκBα degradation in a PPARγ-independent manner. Synovial fibroblasts were pretreated with PPARγ antagonist GW9662 (3 μM) and 15d-PGJ2 for 60 and 30 min, respectively, and TNF-α was then administered for another 6 h. A,B: Semi-quantitative RT-PCR analysis showed that pretreatment with GW9662 alone did not affect MMP-13 expression by itself (n = 5). In addition, pretreatment with GW9662 did not significantly antagonize the inhibitory effects of 15d-PGJ2 on TNF-α-induced MMP-13 mRNA expression. C: ELISA analysis showed that 15d-PGJ2 significantly inhibited TNF-α-induced MMP-13 release, which was not antagonized by GW9662. D: Using real-time PCR analysis, GW9662 at concentrations of 3 and 10 μM could not antagonize the inhibitory effects of 15d-PGJ2 on TNF-α-induced MMP-13 gene expression (n = 5). E: Synovial fibroblasts were pretreated with GW9662 (3 and 10 μM) and 15d-PGJ2 (3 μM) for 30 min and then exposed to TNF-α (10 ng/ml) for another 10 min. Immunoblotting showed that treatment of TNF-α enhanced the phosphorylation of IKKα/β and IκBα and the degradation of IκBα. Pretreatment of 15d-PGJ2 inhibited these TNF-α-induced effects and GW9662 did not antagonize the inhibitory action of 15d-PGJ2 (n = 4). Data are presented as mean ± SEM. $^{*}P < 0.05$ as compared with control group. $^{\#}P < 0.05$ as compared with TNF-α stimulation alone.

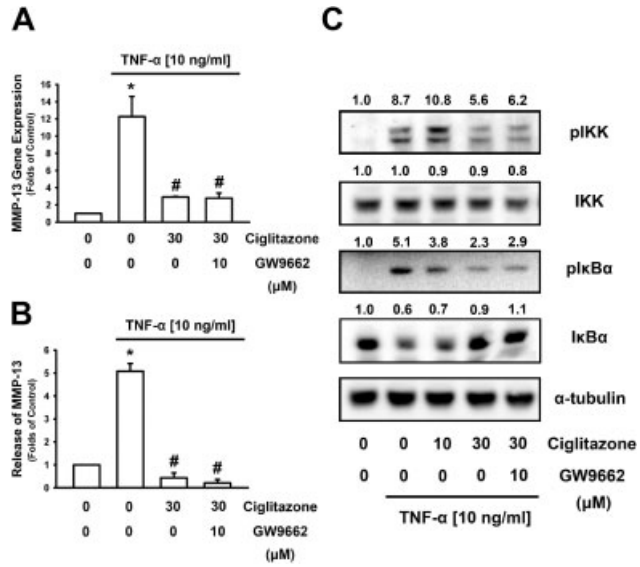

Fig. 5. Ciglitazone inhibits TNF-α-induced MMP-13 by abrogating TNF-α-induced NF-κB activation in a PPAR-γ-independent manner. Synovial fibroblasts were pretreated with PPARγ antagonist GW9662 (10 μM) and ciglitazone for 60 and 30 min, respectively, and TNF-α was then administered for another 6 h. A,B: Quantitative RT-PCR and ELISA analysis showed that pretreatment with GW9662 (10 μM) did not significantly antagonize the inhibitory effects of ciglitazone on TNF-α-induced MMP-13 mRNA expression or protein release (n = 3–4). C: Synovial fibroblasts were incubated with GW9662 (10 μM) and ciglitazone (10–30 μM) for 30 min and then exposed to TNF-α (10 ng/ml) for another 10 min. Immunoblotting showed that pretreatment of ciglitazone (30 μM) significantly antagonized TNF-α-induced phosphorylation of IKKα/β and IκBα and IκBα degradation. The inhibitory effects of ciglitazone were not affected by pretreatment with GW9662 (n = 3). Data are presented as mean ± SEM. $^{*}P < 0.05$ as compared with control group. $^{\#}P < 0.05$ as compared with TNF-α stimulation alone.

TNF-α-induced MMP-13 also through a PPARγ-independent manner. Pretreatment of ciglitazone at 30 μM decreased TNF-α-induced IKK phosphorylation, IκBα phosphorylation, and degradation (Fig. 5C). It has been reported that ciglitazone can inhibit the phosphorylation of IκBα and degradation of IκBα in lung tissue or in pancreatic beta cells (Zingarelli et al., 2003; Kim et al., 2007). However, the interaction mechanism between ciglitazone and IKK activation is still unclear. To further examine how ciglitazone modulates the activation of IKK, the effect of ciglitazone on the modulation of MAPK was explored. The p38-MAPK pathway was reported to be required for TNF-α-induced NF-κB trans-activation (Vanden Berghe et al., 1998) and p38-MAPK is involved in the TNF-α-induced activation of IKKα/β (Chen et al., 2001; de Alvaro et al., 2004). Figure 6A showed that treatment of TNF-α (10 ng/ml) quickly phosphorylated p38-MAPK and ciglitazone but not 15d-PGJ2 abrogated the activation of p38-MAPK. To confirm the relationship between p38-MAPK and NF-κB activation, p38-MAPK specific inhibitor SB203580 was used. Pretreatment of SB203580 decreased TNF-α-induced IKK phosphorylation (Fig. 6B) and pretreatment of SB203580 partially decreased TNF-α-induced NF-κB p50 and p65 translocation from cytosol to nucleus (Fig. 6C). In addition, SB203580 inhibited TNF-α-induced mRNA expression (Fig. 6D) and protein release of MMP-13 (Fig. 6E) in synovial fibroblasts. Effects of dominant negative mutant of p38 was also examined, treatment of dominant negative mutant can also decrease TNF-α-induced p38-MAPK activation (Supplementary Fig. 2A) and TNF-α-induced MMP-13 mRNA expression (42.2 ± 9.3%, Supplementary Fig. 2B). These results indicate that p38-MAPK was involved in the inhibitory action of ciglitazone on NF-κB activation.

Discussion

Type II collagen is the major fibrillar interstitial collagen in cartilage and is resistant to most proteinase because of its triple-helical structure. However, the MMPs such as classical collagenases including collagenase-1 (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13) are able to degrade type II collagen (Takaishi et al., 2008). These MMPs can be expressed by cells of cartilage, synovium, and infiltrated

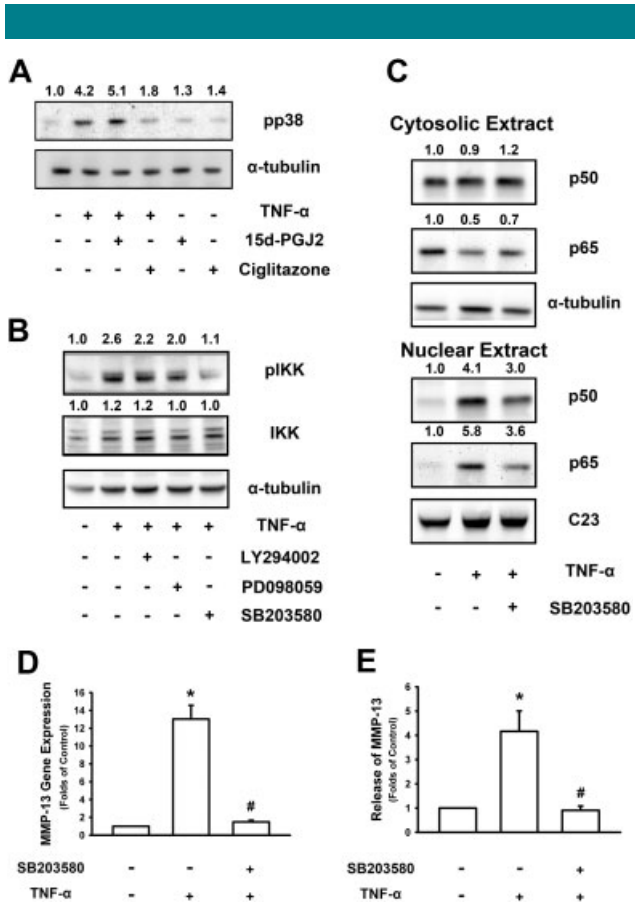

Fig. 6. Inhibition of TNF-α-induced p38-MAPK activation by ciglitazone. A: Synovial fibroblasts were incubated with 15d-PGJ2 (3 μM) or ciglitazone (30 μM) for 30 min and then exposed to TNF-α (10 ng/ml) for another 10 min. Treatment of TNF-α increased the phosphorylation of p38-MAPK. Ciglitazone but not 15d-PGJ2 attenuated the phosphorylation of P38-MAPK (n = 3). B: Pretreatment of specific P38-MAPK inhibitor SB203580 (10 μM) but not PI3K inhibitor LY294002 (20 μM) or ERK1/2 inhibitor PD098059 (20 μM) for 30 min significantly inhibited TNF-α-induced IKK phosphorylation (n = 4). C: Cells were pretreated with SB203580 for 30 min and then incubated with TNF-α for another 30 min. Pretreatment of SB203580 inhibited the TNF-α-induced nuclear translocation of NF-κB subunits of p50 and p65 (n = 3). D,E: Quantitative RT-PCR and ELISA analysis showed that pretreatment of SB203580 antagonized TNF-α-induced mRNA expression and protein release of MMP-13 (n = 3). Data are presented as mean ± SEM. $^{*}P < 0.05$ as compared with control group. $^{\#}P < 0.05$ as compared with TNF-α stimulation alone.

inflammatory cells (Brennan and McInnes, 2008). MMP-1 is reported to be expressed in synovial fibroblasts and chondrocytes (Zayed et al., 2008; Noh et al., 2009) and it has been mentioned that the systemic levels of MMP-1 in RA patients are associated with the development of joint erosions (Cunnane et al., 2001). MMP-8 is also reported to be expressed in macrophages, neutrophils, fibroblasts, and chondrocytes (Hanemaaijer et al., 1997; Yoshihara et al., 2000). In addition, serum MMP-8 levels in OA or RA patients are higher than in control patients and may play a part of the cartilage degradation (Tchetverikov et al., 2004). However, of these three collagenases, MMP-13 is the most efficient one to degrade type II collagen (Billinghurst et al., 1997) and it can also cleave other components of cartilage including type IX collagen and aggrecan (Mitchell et al., 1996; Mercuri et al., 2000). MMP-13 is reported to play a pivotal role in cartilage destruction and is rarely detected in normal human tissue but is expressed by chondrocytes and synovial cells in OA or RA patients (Takaishi et al., 2008). In experimental OA model, it is reported that transgenic mice constitutively expressing MMP-13 exhibit OA changes under physiological conditions, indicating that MMP-13 is involved in cartilage destruction in OA (Neuhold et al., 2001). In RA patients, MMP-13 is reported to be one of the most important regulators in joint destruction (Distler et al., 2005) and it has been shown that intra-articular overexpression of MMP-13 by adenovirus can induce inflammatory arthritis in mice (Joronen et al., 2004). Moreover, oral application of specific MMP-13 inhibitors has been shown to ameliorate the symptoms in two different animal models of RA (Jungel et al., 2010).

The present study shows that treatment of 15d-PGJ2 and ciglitazone significantly inhibited TNF-α-induced MMP-13 mRNA expression and protein production in human synovial fibroblasts. However, the inhibitory actions of 15d-PGJ2 and ciglitazone could not be antagonized by the pretreatment of specific PPARγ antagonist GW9662. It has been reported that modulation of NF-κB signaling is mainly involved in the PPARγ-independent pathways of 15d-PGJ2 and TZDs. Rossi et al. (2000) demonstrate that 15d-PGJ2 can covalently bind to IκB kinase (IKK), inhibiting its function and therefore the activation of NF-κB. 15d-PGJ2 is also reported to inhibit NF-κB binding to DNA in a PPARγ-independent manner, via alkylation of a conserved cysteine residue located in NF-κB DNA-binding domain (Straus et al., 2000). In addition, the induced production of cytokines and MMPs including MMP-1, MMP-3, and MMP-13, is NF-κB-dependent in synovial fibroblasts (Bondeson et al., 2007). Our results show that 15d-PGJ2 and ciglitazone inhibited TNF-α-induced MMP-13 expression via the inhibition of NF-κB signaling. Treatment of 15d-PGJ2 and ciglitazone inhibited the phosphorylation of IKKα/β following TNF-α stimulation. 15d-PGJ2 and ciglitazone also attenuated the downstream steps of IKK, such as the phosphorylation and degradation of IκBα and the nuclear translocation of NF-κB p50 and p65 subunits. However, there is no evidence to show how ciglitazone modulates the activation of IKK. To find out the possible mechanisms how ciglitazone inhibits the induction of IKK by TNF-α, we examined the roles of p38-MAPK. In synovial membrane, mitogen-activated protein kinases (MAPK) including p38 and ERK are quickly activated by TNF-α stimulation (Gortz et al., 2005). Activation of MAPK leads to the activation of IκB kinase (IKK) and the phosphorylation of IκBα (de Alvaro et al., 2004), resulting in the degradation of IκBα and the nuclear translocation of NF-κB. Several reports indicate that p38-MAPK is essential for TNF-α-induced IKK activation (Chen et al., 2001; de Alvaro et al., 2004) or oxidative stress-induced IKK activation (Lee et al., 2005). Furthermore, TZD compounds of pioglitazone and ciglitazone are reported to inhibit inflammatory response via modulating p38-MAPK activity (Syrovets et al., 2002; Ji et al., 2010). Moreover, Shibata et al. (2008) demonstrated that pioglitazone inhibits the activation of p38-MAPK and degradation of IκBα via a PPARγ-independent manner. Here we found that treatment of ciglitazone but not 15d-PGJ2 significantly inhibited the activation of p38-MAPK in response to TNF-α stimulation (Fig. 6A) and treatment of p38-MAPK inhibitor SB203580 exerted the similar inhibitory actions on IKK and NF-κB translocation and MMP-13 expression (Fig. 6B–E). Transfection of p38-MAPK dominant negative mutant also decreased TNF-α-induced MMP-13 mRNA expression (Supplementary Fig. 2). It has also been reported that inhibition of p38-MAPK by SB203580 markedly antagonized TNF-α-induced MMP-13 expression in keratinocytes (Johansson et al., 2000). Here we also found that p38-MAPK was involved in TNF-α-induced MMP-13 in synovial fibroblasts. Taken together, both prostaglandin 15d-PGJ2 and synthetic TZD compound

ciglitazone showed potential anti-inflammation by abrogating the activation of NF-κB in synovial fibroblasts.

NSAIDs including indomethacin and diclofenac act through the inhibition of COX and are widely used for the treatment of pain and inflammation in arthritis. However, a growing evidence shows that NSAIDs exert chondrotoxic effects in OA animal model (Serni et al., 1999) and accelerate radiographic progression of OA in patients with hip and knee OA (Reijman et al., 2005). While 15d-PGJ2 and its upstream product PGD2 possess anti-inflammatory and chondroprotective actions (Zayed et al., 2008), the inhibition of biosynthesis of endogenous PGD2 and 15d-PGJ2 by NSAIDs may be related to their deleterious effect on cartilage. In carrageenan-induced pleurisy in rats, COX-2 protein expression peaks initially at 2 h associated with inflammatory PGE2 synthesis in leukocytes (Gilroy et al., 1999). However, a greater secondary expression of COX-2 at 48 h is associated with the increase of anti-inflammatory prostaglandins of PGD2 and 15d-PGJ2. Therefore, there is a therapeutic limitation in the treatment of inflammatory joint diseases by the inhibition of all kinds of prostaglandin (Zayed et al., 2008). On the other hand, TZDs may be a potential drug candidate for the treatment of inflammatory diseases including OA and RA. Bongartz et al. (2005) show that treatment of pioglitazone ameliorates the disease in patients with psoriatic arthritis. Moreover, piogliatzone is now undergoing phase III clinical trial for patients with RA.

In conclusion, we demonstrate that both 15d-PGJ2 and ciglitazone can suppress TNF-α-induced MMP-13 expression in human synovial fibroblasts. As shown in Figure 7, at the initial stage following TNF-α stimulation, both 15d-PGJ2 and ciglitazone quickly attenuated the activation of NF-κB by inhibiting the activation of IKKα/β via a PPARγ-independent manner and modulation of p38-MAPK is involved in the inhibitory effects of ciglitazone. These findings suggest that 15d-PGJ2 and ciglitazone are potential therapeutic agents to reduce the release of MMP-13 in RA and OA.

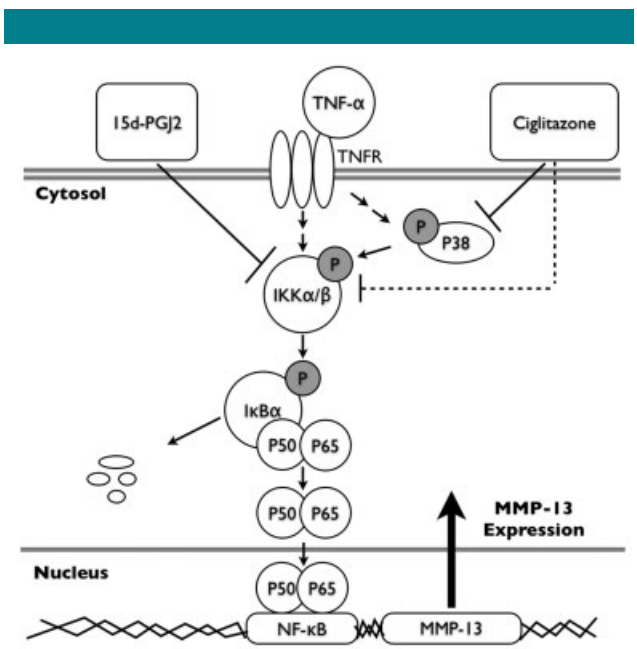

Fig. 7. Schematic diagram of the signaling pathways involved in the inhibition of TNF-α-induced MMP-13 expression by 15d-PGJ2 and ciglitazone. TNF-α enhanced MMP-13 production via NF-κB signaling pathways in synovial fibroblasts. 15d-PGJ2 inhibited the TNF-α-induced MMP-13 expression via a PPARγ-independent manner and mainly acted by direct inhibition of IKKα/β activation. Ciglitazone inhibited TNF-α-induced MMP-13 release via inhibiting IKKα/β activation and modulation of p38-MAPK signaling is involved in this inhibitory effect.

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