Expression and Regulation of Microsomal Prostaglandin E Synthase-1 in Human Osteoarthritic Cartilage and Chondrocytes

XINFANG LI, HASSAN AFIF, SARANETTE CHENG, JOHANNE MARTEL-PELLETIER, JEAN-PIERRE PELLETIER, PIERRE RANGER, and HASSAN FAHMI

ABSTRACT. ***Objective.*** Elevated production of prostaglandin E_2 (PGE_2) plays an important role in the pathogenesis of arthritis. Recently, an inducible microsomal prostaglandin E synthase-1 (mPGES-1) was identified. This enzyme is functionally coupled with cyclooxygenase-2 (COX-2) and converts the COX product PGH_2 to PGE_2. We analyzed expression of mPGES-1 in human normal and osteoarthritic (OA) cartilage and determined the effect of different inflammatory agonists on the expression of mPGES-1 in OA chondrocytes.

Methods. Expression of mPGES-1 mRNA and protein in cartilage was determined by quantitative real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry, respectively. OA chondrocytes were treated with different inflammatory agents, and mPGES-1 protein expression was evaluated by Western blot. Activation of the mPGES-1 promoter was assessed in transient transfection experiments.

Results. Levels of mPGES-1 mRNA and protein were markedly elevated in OA versus normal cartilage. Treatment of chondrocytes with interleukin 1ß (IL-1ß) induced expression of mPGES-1 protein in a dose- and time-dependent manner. This appears to occur at the transcriptional level, as IL-1ß induced expression of mPGES-1 mRNA and the activity of this gene promoter. Tumor necrosis factor-α (TNF-α) and IL-17 also upregulated expression of mPGES-1 protein and displayed a synergistic effect with IL-1ß. Peroxisome proliferator-activated receptor-γ ligands, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and troglitazone, inhibited IL-1ß-induced mPGES-1 protein expression, an effect that was reversed by exogenous PGE_2.

Conclusion. Our study shows that mPGES-1 expression is upregulated in OA versus normal cartilage and that proinflammatory cytokines increased mPGES-1 expression in chondrocytes. These data suggest that mPGES-1 may prove to be an interesting therapeutic target for controlling PGE_2 synthesis. (J Rheumatol 2005;32:887–95)

Key Indexing Terms:
MICROSOMAL PROSTAGLANDIN E SYNTHASE-1 CARTILAGE
CHONDROCYTES OSTEOARTHRITIS

Biochemical, genetic, and clinical evidence indicates that prostaglandin E_2 (PGE_2) plays a critical role in inflammation and in the pathophysiology of articular joint diseases, such as rheumatoid arthritis (RA) and osteoarthritis (OA). For example, arthritic joint tissues produce large quantities of PGE_2[1]. Treatment with neutralizing anti-PGE_2 antibodies prevents acute and chronic inflammation in a rat adjuvant arthritis model[2]. More direct evidence for the role of PGE_2 in arthritis has been provided by gene targeting studies. Genetic disruption of either the PGE_2 receptor EP4[3] or cyclooxygenase-2 (COX-2)[4], one of the key enzymes in PGE_2 biosynthesis, reduced incidence and severity of collagen-induced arthritis in mice. These animals showed reduced inflammation and less cartilage and bone destruction. The role of PGE_2 in arthritis is also supported by effective suppression of pain and inflammatory responses in arthritis by nonsteroidal antiinflammatory drugs (NSAID) that reduce PGE_2 biosynthesis[5,6].

Chondrocytes are a major source of PGE_2 in the joint; the production of this prostanoid can be induced by proinflammatory cytokines, mitogens, mechanical stress, and trauma[5,7,8]. The synthesis of PGE_2 from arachidonic acid (AA) requires 2 enzymes acting sequentially. Cyclooxygenases catalyze the conversion of AA to the intermediate prostanoid

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PGH_2. Two isoforms of the COX enzyme have been identified: COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by various stimuli including lipopolysaccharides, growth factors, and proinflammatory cytokines (reviewed in[5,9]). Subsequently, PGE synthase (PGES) converts COX-derived PGH_2 into PGE_2. At least 3 distinct PGES isoforms have been identified[10]. Cytosolic PGES (cPGES), which is identical to the heat shock protein 90-associated protein p23, is ubiquitously and constitutively expressed, and displays functional coupling with COX-1. In contrast, microsomal PGES-1 (mPGES-1), originally designated microsomal glutathione S-transferase 1-like 1, is an inducible enzyme that exhibits preferential functional coupling with COX-2. The most recently identified isoform, mPGES-2, is ubiquitously expressed in diverse tissues, but its function and regulation remain obscure[10].

The upregulation of mPGES-1 expression has been reported in conditions in which PGE_2 has been implicated, such as arthritis[11], and studies with mPGES-1-deficient mice have shown that induced PGE_2 synthesis is largely dependent on this enzyme[12,13]. Proinflammatory cytokines interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) have been shown to induce mPGES-1 expression in several tissues and cell types, including synovial fibroblasts and osteoblasts[14,15]. However, little is known about the expression and regulation of mPGES-1 in cartilage.

To understand the regulation of PGE_2 production in joint tissues, we analyzed mPGES-1 expression in normal and OA cartilage. Further, we explored the effect of different inflammatory agonists on the expression of mPGES-1 in OA chondrocytes.

MATERIALS AND METHODS

Reagents. Recombinant human (rh) IL-1ß was obtained from Genzyme (Cambridge, MA, USA), rhTNF-α and rhIL-17 were from R&D Systems (Minneapolis, MN, USA). 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), troglitazone, Wy14643, and PGE_2 were from Cayman Chemical Co. (Ann Arbor, MI, USA). BRL 49653 was from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal calf serum (FCS), and TRIzol® reagent were from Invitrogen (Burlington, ON, Canada). All other chemicals were purchased from either Bio-Rad (Mississauga, ON, Canada) or Sigma-Aldrich Canada (Oakville, ON, Canada).

Specimen selection and chondrocyte culture. Human normal cartilage (femoral condyles) was obtained at necropsy, within 12 hours of death, from donors with no history of arthritic disease (n = 7, mean ± SEM age 61 ± 15 yrs). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically. Only those with no alterations were processed further. Human OA cartilage was obtained from patients undergoing total knee replacement (n = 25, mean ± SEM age 64 ± 14 yrs). All OA patients were diagnosed based on criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA. At the time of surgery, patients had symptomatic disease requiring medical treatment in the form of NSAID or selective COX-2 inhibitors. Patients who had received intraarticular injections of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion as described[16]. Briefly, this consisted of 2 mg/ml pronase for 1 h followed by 1 mg/ml collagenase for 6 h (type IV; Sigma-Aldrich) at 37°C in DMEM and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS. At confluence, the chondrocytes were detached, seeded at high density, and allowed to grow in DMEM, supplemented as above. The culture medium was changed every second day, and 24 h before the experiment the cells were incubated in fresh medium containing 0.5% FCS. Only first-passage chondrocytes were used.

Immunohistochemistry. Cartilage specimens were processed for immunohistochemistry as described[16]. Specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) of paraffin-embedded specimens were deparaffinized in toluene, and dehydrated in a graded series of ethanol. The specimens were then preincubated with chondroitinase ABC (0.25 U/ml in phosphate buffered saline, PBS, pH 8.0) for 60 min at 37°C, followed by 30 min incubation with Triton X-100 (0.3%) at room temperature. Slides were then washed in PBS followed by 2% hydrogen peroxide/methanol for 15 min. They were further incubated for 60 min with 2% normal serum (Vector Laboratories, Burlingame, CA, USA) and overlaid with primary antibody for 18 h at 4°C in a humidified chamber. The antibody was a rabbit polyclonal anti-human mPGES-1 (Cayman), used at 10 µg/ml. Each slide was washed 3 times in PBS pH 7.4 and stained using the avidin-biotin complex method (Vectastain ABC kit; Vector). The color was developed with 3,3'-diaminobenzidine (DAB; Vector) containing hydrogen peroxide. Slides were counterstained with eosin. The specificity of staining was evaluated by using antibody that had been preadsorbed (1 h, 37°C) with a 20-fold molar excess of the specific corresponding peptide, and by substituting the primary antibody with non-immune rabbit IgG (Chemicon, Temecula, CA, USA; used at the same concentration as the primary antibodies). The evaluation of positive-staining chondrocytes was performed using our published method[16]. For each specimen, 6 microscopic fields were examined under 40× magnification. The total number of chondrocytes and the number of chondrocytes staining positive were evaluated and results were expressed as the percentage of chondrocytes staining positive (cell score).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from homogenized cartilage or stimulated chondrocytes was isolated using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA), dissolved in diethylpyrocarbonate (DEPC)-treated-H_2O, and stored at –80°C until use. One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, ON, Canada) as detailed in the manufacturer's guidelines. One-fiftieth of the reverse transcriptase reaction was analyzed by real-time quantitative PCR as described below. The following primers were used: mPGES-1, sense 5'-GAA GAA GGC CTT TGC CAA C-3', antisense 5'-GGA AGA CCA GGA AGT GCA TC-3'; cPGES, sense 5'-GCA AAG TGG TAC GAT CGA AGG-3', antisense 5'-TGT CCG TTC TTT TAT GCT TGG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-CAG AAC ATC ATC CCT GCC TCT-3', antisense 5'-GCT TGA CAA AGT GGT CGT TGA G -3'.

Real-time quantitative PCR. Quantitative PCR analysis was performed in a total volume of 50 µl containing template DNA, 200 nM of sense and antisense primers, 25 µl of SYBR® Green master mix (Qiagen, Mississauga, ON, Canada) and uracil-N-glycosylase (UNG, 0.5 Unit; Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 min (UNG reaction) and at 95°C for 10 min (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products

was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak, indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems). Preliminary experiments showed that the amplification efficiency of cPGES, mPGES-1, and GAPDH was similar.

Relative amounts of mRNA in normal and OA cartilage were determined using the standard curve method. Serial dilutions of internal standards (plasmids containing cDNA of target genes) were included in each PCR run, and standard curves for the target gene and for GAPDH were generated by linear regression using log (C_T) versus log (cDNA relative dilution). The C_T were then converted to number of molecules. Relative mRNA expression in cultured chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control (unstimulated cells) from the ΔC_T value of each treatment. Fold changes compared with the control were then determined by raising 2 to the $\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on 2 separate occasions for each independent experiment.

PGE_2 assay. At the end of the incubation period, the culture medium was collected and stored at –80°C. Levels of PGE_2 were determined using a PGE_2 enzyme immunoassay kit from Cayman Chemical. The detection limit and sensitivity was 9 pg/ml. All assays were performed in duplicate.

Plasmids and transient transfection. The luciferase reporter constructs pmPGES-1-Luc and pcPGES-Luc were kindly provided by Dr. T.J. Smith (University of California, Los Angeles)[17]. pmPGES-1-Luc contains a 510-bp fragment of the human mPGES-1 promoter spanning 538 to –28. pcPGES-Luc contains a 1824-bp fragment of the human cPGES promoter spanning 1893 to –69. ß-Galactosidase reporter vector under the control of SV40 promoter (pSV40-ß-galactosidase) was from Promega (Madison, WI, USA). Transient transfection experiments were performed using FuGene-6 (1 µg DNA :4 µl FuGene 6) (Roche Applied Science, Laval, Quebec, Canada) according to the manufacturer's recommended protocol. Briefly, chondrocytes were seeded and grown to 50–60% confluence. The cells were transfected with 1 µg of the reporter construct and 0.5 µg of the internal control pSV40-ß-galactosidase (Promega). Six hours later, the medium was replaced with DMEM containing 1% FCS. The next day, the cells were treated for 18 h with or without IL-1ß. After harvesting, luciferase activity was determined and normalized to ß-galactosidase activity[16].

Western blot analysis. Chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Ten micrograms total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with primary antibodies and washed with a Tris buffer [Tris-buffered saline (TBS) pH 7.5, with 0.1% Tween 20]. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak Ltd., Rochester, NY, USA).

Statistical analysis. Data are expressed as the mean ± SEM. Statistical significance was assessed by the 2-tailed Student t test. p values less than 0.05 were considered significant.

RESULTS

Increased expression of mPGES-1 in OA cartilage. We first analyzed levels of mPGES-1 mRNA in normal (n = 7) and OA (n = 8) cartilage using real-time quantitative RT-PCR. As shown in Figure 1A, levels of mPGES-1 expression in cartilage from OA patients were 2.8-fold higher compared with those from normal cartilage ($p < 0.05$). In contrast to mPGES-1, there was no statistically significant difference in the level of cPGES expression between OA and normal cartilage (Figure 1B).

To examine whether mPGES-1 protein is also expressed in cartilage, normal (n = 5) and OA (n = 5) cartilage were processed for immunohistochemical analysis. In normal cartilage, the positive immunostaining for mPGES-1 was located only in a few chondrocytes in the superficial layer (mean ± SEM 9.1 ± 0.6%; Figure 2A). In contrast, the cell score was higher in OA cartilage (mean ± SEM 24.4 ± 1.8%) than it was in normal cartilage (Figure 2B). Statistical evaluation of the cell score for mPGES-1 indicated significant differences between normal and OA cartilage ($p < 0.001$). The specificity of staining was confirmed by immunohistochemical staining using an anti-mPGES-1 antibody that had been preadsorbed with the peptide antigen (Figure 2C) or nonimmune rabbit IgG at the same concentration (data not shown). These observations demonstrate an upregulation of mPGES-1 expression in OA cartilage.

Induction of mPGES-1 expression by IL-1ß in chondrocytes. To explore the mechanisms by which mPGES-1 is regulated in cartilage, we examined the effect of IL-1ß, a key mediator in the pathogenesis of arthritis, on both the expression of mPGES-1 and the production of PGE_2 by OA chondrocytes. Under basal culture conditions, OA chondrocytes express low levels of mPGES-1 protein. Treatment with IL-1ß (100 pg/ml) enhanced expression of mPGES-1 protein in a time-dependent manner (Figure 3A). The level of mPGES-1 expression started to increase 6 h post-stimulation with IL-1ß and reached maximum at 24 h. The increased expression of mPGES was sustained for at least 48 h. The induction of mPGES-1 protein expression was also dose-dependent (Figure 3B). The level of mPGES-1 was increased at IL-1ß concentrations as low as 1 pg/ml and reached a maximum at 100 pg/ml. In concert with the effects on mPGES-1 expression, IL-1ß stimulated PGE_2 production in a time- and dose-dependent manner (Figure 3, lower panels). In contrast, the level of cPGES was not altered as a consequence of IL-1ß treatment.

IL-1ß induced mPGES-1 expression at the transcriptional level. To further elucidate the mechanism responsible for the upregulation of mPGES-1 protein, we analyzed the effect of IL-1ß on the expression of mPGES-1 mRNA. Chondrocytes were treated with increasing concentrations of IL-1ß for 12 h, and specific mRNA for mPGES-1 and cPGES were quantified by real-time RT-PCR. IL-1ß-induced changes in gene expression were expressed as -fold over control (untreated

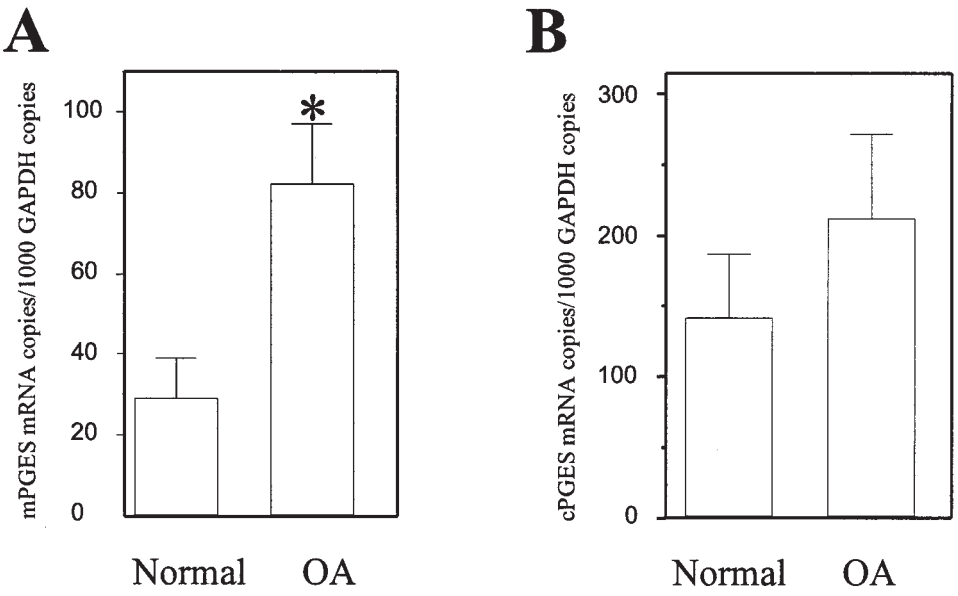


Figure 1. Relative expression of mPGES-1 (A) and cPGES (B) in normal and OA human cartilage. RNA was extracted from normal (n = 7) and OA (n = 8) cartilage, reverse transcribed into cDNA, and processed for real-time PCR. The threshold cycle values were converted to the number of molecules, as described under Materials and Methods. Data were expressed as copies of gene mRNA detected per 1000 GAPDH copies. *p < 0.05 versus normal samples.

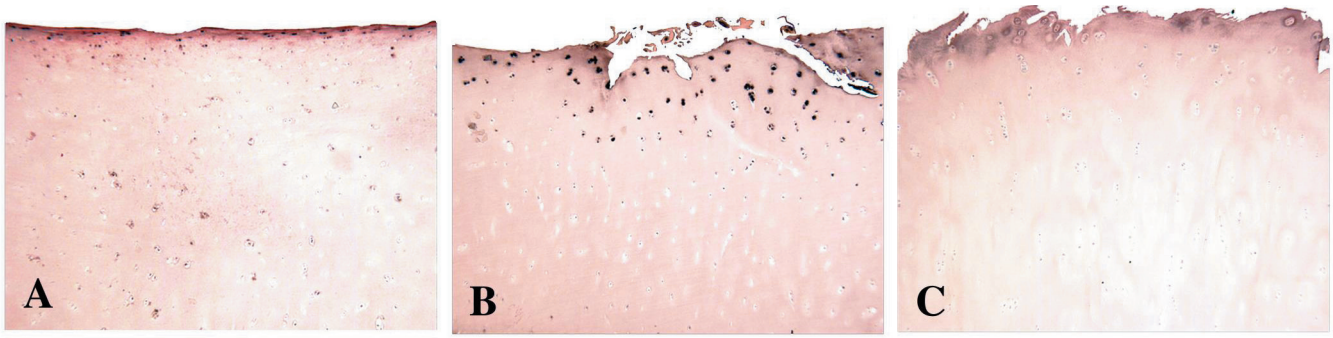


Figure 2. Representative immunostaining of human normal (n = 5) (A) and OA cartilage (n = 5) (B) for mPGES-1. C. OA specimens treated with anti-mPGES-1 antibody that was preadsorbed with a 20-fold molar excess of the blocking mPGES-1 peptide (control for staining specificity). Results are representative of 3 separate experiments.

cells) after normalizing to the internal control GAPDH. Results showed that IL-1ß induced a dose-dependent increase in mPGES-1 mRNA expression, but had no effect on the levels of cPGES mRNA (Figure 4A).

To determine whether changes in mRNA levels can be ascribed to changes in promoter activity, chondrocytes were transiently transfected with the human mPGES-1 or cPGES promoter-luciferase reporter genes. Treatment of transfected cells with increasing concentrations of IL-1ß led to a dose-dependent increase of the mPGES-1 promoter activity (Figure 4B). In contrast, IL-1ß had no significant effect on the cPGES promoter activity. These data are consistent with the regulation of mPGES-1 expression by IL-1ß being at the level of transcription.

Effect of the combination of IL-1ß, TNF-α, and IL-17 on mPGES-1 expression. The proinflammatory cytokines TNF-α and IL-17 are also implicated in the pathogenesis of arthritis and are potent inducers of COX-2 expression and PGE_2 production in articular chondrocytes[18,19]. Therefore, we examined the effect of both cytokines on mPGES-1 expression. Chondrocytes were stimulated with increasing concentrations of IL-17 (0.5–1000 ng/ml) or TNF-α (0.5–10,000 pg/ml) for 24 h, and mPGES-1 protein expression was evaluated by Western blot analysis. As shown in Figure 5A and 5B, treatment with TNF-α or IL-17 induced mPGES-1 expression and PGE_2 production in a dose-dependent manner. At optimal concentrations the effect of IL-1ß (100 pg/ml) was more potent than that of IL-17 (1000 ng/ml) or TNF-α (10 ng/ml).

Previous studies have demonstrated that low concentra-

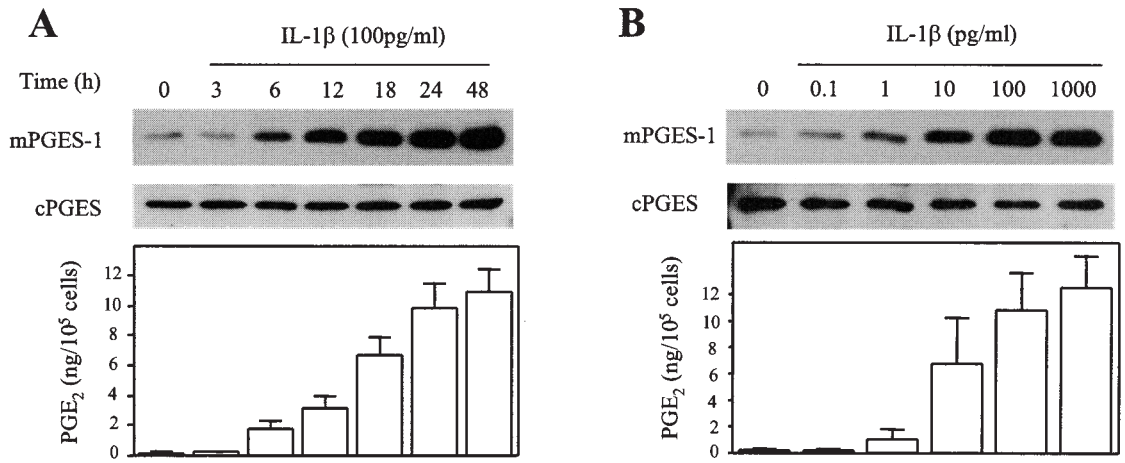
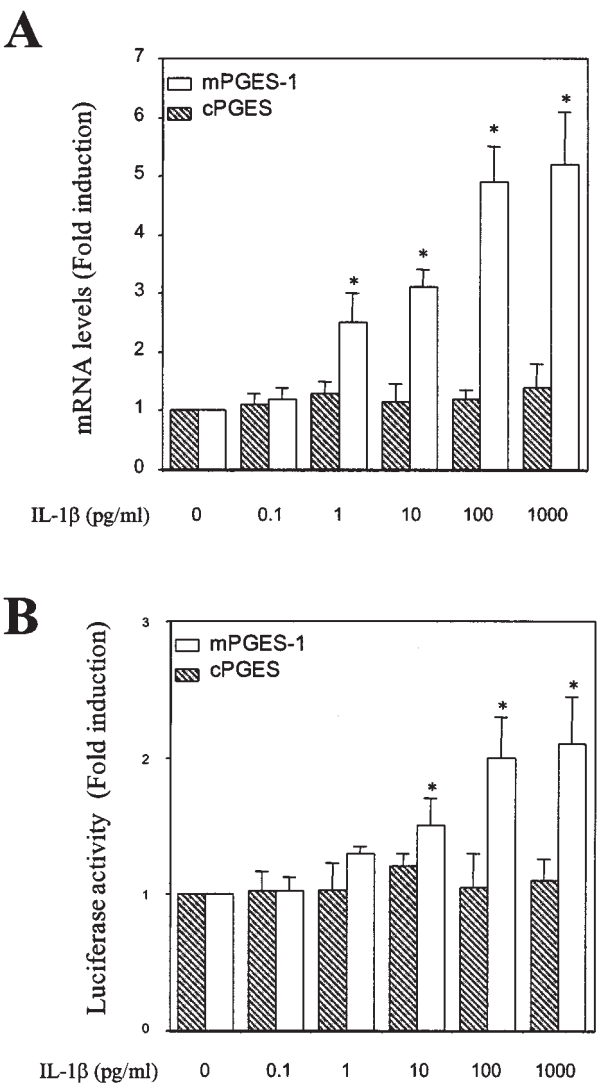


Figure 3. Effect of IL-1ß on mPGES-1 protein expression in OA chondrocytes. A. Cells were treated with 100 pg/ml IL-1ß for the indicated time periods. B. Chondrocytes were treated with increasing concentrations of IL-1ß for 24 h. Cell lysates were prepared and analyzed for mPGES-1 protein by Western blotting (upper panels). Blots were stripped and reprobed with a specific anti-cPGES antibody (middle panels). The blots are representative of similar results obtained from 4 independent experiments. In the lower panels, conditioned media was collected and analyzed for PGE_2. Results are expressed as the mean ± SEM of 3 independent experiments. *$p < 0.05$ compared with unstimulated cells.



tions of IL-1ß, TNF-α, and IL-17 were synergistic in a number of systems[20-24]. Therefore, we examined the effect of different combinations of these cytokines on mPGES-1 expression and PGE_2 production. At a lower concentration, IL-1ß (0.1 pg/ml), IL-17 (0.5 ng/ml), and TNF-α (0.5 pg/ml) alone had little or no effect on mPGES-1 expression and PGE_2 production. Each combination of 2 cytokines resulted in a marked increase of mPGES-1 expression versus either cytokine alone, indicating a synergistic effect (Figure 5C). The combination of either IL-1ß and TNF-α or IL-1ß and IL-17 resulted in a greater effect than IL-17 and TNF-α. In addition, the combination of 3 cytokines led to a more potent effect versus combination of any 2 of the cytokines (Figure 5C). These findings indicate that low levels of cytokines can act in combination to upregulate the expression of mPGES-1.

Peroxisome proliferator-activated receptorγ (PPARγ) ligands inhibited IL-1ß-induced mPGES-1 expression. PPARγ ligands have been shown to inhibit the expression of a number of genes involved in the pathogenesis of arthritis[25]. To assess the effect of these molecules on mPGES-1 expression

Figure 4. IL-1ß induced mPGES-1 expression at the transcriptional level. A. Chondrocytes were treated with increasing concentrations of IL-1ß for 12 h. Total RNA was isolated; reverse transcribed into cDNA; and mPGES-1, cPGES, and GAPDH mRNA were quantified using real-time PCR. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. B. Chondrocytes were cotransfected with 1 µg/well of either the mPGES-1 promoter or the cPGES promoter and 0.5 µg/well of the internal control pSV40-—galactosidase, using FuGene 6 transfection reagent. The next day, transfected cells were treated with increasing concentrations of IL-1ß for 18 h. Luciferase activity values were determined and normalized to γ-galactosidase activity. Results are expressed as –fold changes, considering 1 as the value of untreated cells and represent the mean ± SEM of 4 independent experiments. *$p < 0.05$ compared with unstimulated cells.

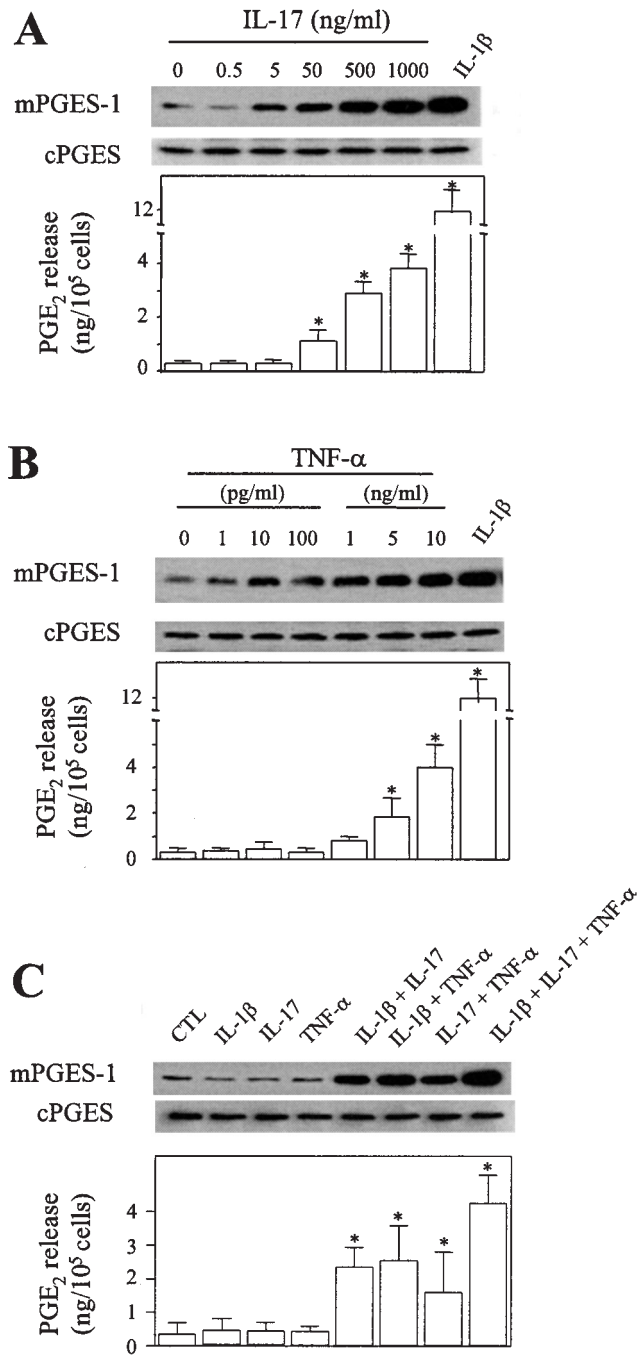


Figure 5. Effect of TNF-α and IL-17 on mPGES-1 protein expression in OA chondrocytes. Cells were treated with increasing concentrations of IL-17 (A), TNF-α (B), or IL-1ß (100 pg/ml). C. cells were treated with IL-1ß (0.1 pg/ml), IL-17 (0.5 ng/ml), TNF-α (0.5 pg/ml), alone or in combination. After 24 h, cell lysates were prepared and analyzed for mPGES-1 and cPGES proteins by Western blotting. The blots are representative of similar results obtained from 4 independent experiments. In the lower panels, conditioned media was collected and analyzed for PGE_2. Results are expressed as the mean ± SEM of 3 independent experiments. *$p < 0.05$ compared with unstimulated cells.

in chondrocytes, we first examined the natural PPARγ ligand, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). Chondrocytes were stimulated with IL-1ß (100 pg/ml) in the absence or presence of increasing concentrations of 15d-PGJ_2, and the expression of mPGES-1 was evaluated by Western blotting. As shown in Figure 6A, 15d-PGJ_2 dose-dependently prevented IL-1ß-induced mPGES-1 expression. Troglitazone, a synthetic and selective PPARγ ligand, also inhibited IL-1ß-induced mPGES-1 expression (Figure 6B). In contrast, the PPARα ligand Wy14643 did not affect IL-1ß-induced mPGES expression (Figure 6C). cPGES expression was not affected by these treatments (Figure 6, lower panels). Taken together, these data suggest that 15d-

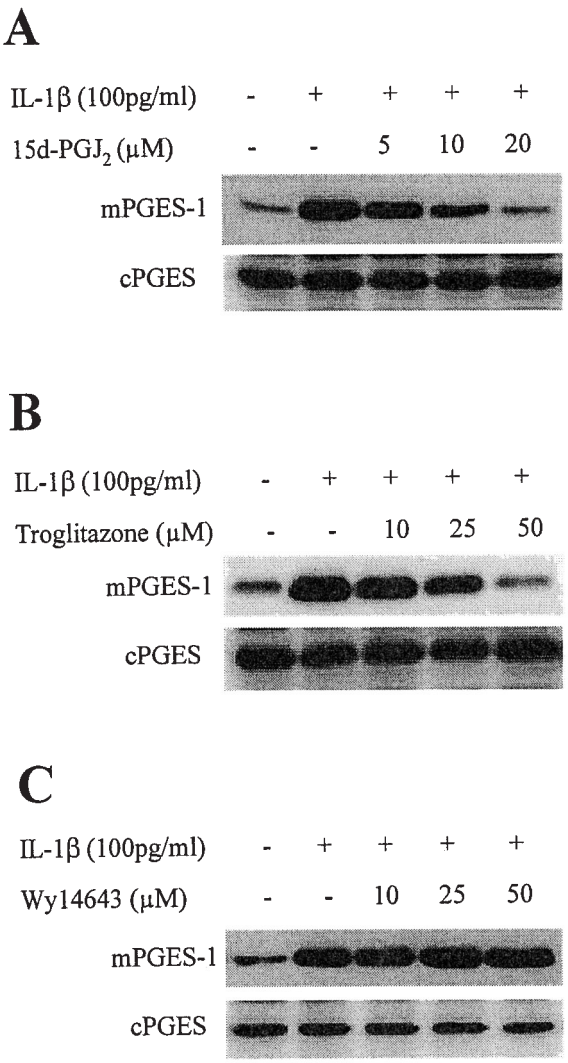


Figure 6. PPARγ ligands inhibited IL-1ß-induced mPGES-1 protein expression. Chondrocytes were pretreated with increasing concentrations of 15d-PGJ_2 (A), troglitazone (B), or Wy14643 (C) for 30 min before incubation in the presence of 100 pg/ml IL-1ß for 24 h. Cell lysates were prepared and analyzed for mPGES-1 protein by Western blotting. In the lower panels, the blots were stripped and reprobed with a specific anti-cPGES antibody. The blots are representative of similar results obtained from 4 independent experiments.

PGJ_2 prevented IL-1ß-induced mPGES-1 expression, at least in part, through a PPARγ-dependent mechanism.

PPARγ ligand-inhibited IL-1ß-induced mPGES-1 expression is alleviated by PGE_2. Next, we evaluated the role of PGE_2, the end product of mPGES-1, in the repressing effect of PPARγ ligands. Chondrocytes were preincubated with increasing concentrations of PGE_2 (0.01–1 μM) for 30 min, prior to the addition of 15d-PGJ_2 (20 μM) or troglitazone (50 μM), and were subsequently stimulated with IL-1ß (100 pg/ml) for 24 h. Western blot analysis revealed that PGE_2 dose-dependently alleviated the suppressive effect of 15d-PGJ_2 (Figure 7A) or troglitazone (Figure 7B) on IL-1ß-induced mPGES-1 expression. Of note, PGE_2 alone had no significant effect on mPGES-1 expression (Figure 7A and 7B, last 3 lines). As expected, the level of cPGES expression was not affected by these treatments.

DISCUSSION

It is well established that increased production of PGE_2 plays a central role in the pathogenesis of arthritis, and inhibitors of PGE_2 synthesis are widely used in the treatment of OA and RA[5,26]. PGE_2 biosynthesis from arachidonic acid is controlled by 2 rate-limiting enzymatic reactions. The first step is catalyzed by COX, which transforms AA into the unstable metabolite PGH_2. The second step is catalyzed by PGES, which converts PGH_2 into PGE_2. Several PGES were identified, among which mPGES-1 has been shown to be functionally coupled with COX-2 and to be upregulated by proinflammatory stimuli in several cell types and tissues[10]. However, little is known about the expression and regulation of mPGES-1 in human cartilage.

In our study, we showed that human cartilage also expresses mPGES-1. Using real-time quantitative RT-PCR, we found that mPGES-1 mRNA expression was elevated in OA cartilage when compared with normal cartilage. Immunohistochemical analysis corroborates these findings, showing higher levels of mPGES-1-positive cells in OA versus normal cartilage. This is similar to results of 2 recent studies showing that mPGES-1 is overexpressed in OA cartilage[27,28]. In our studies, the positive immunoreactive staining for mPGES-1 was located mainly in chondrocytes in the superficial layers. Interestingly, IL-1ß, one of the most important mediators involved in articular inflammation and degradation processes, has been shown to accumulate in these zones[29,30]. This suggests that IL-1ß could be a key mediator of mPGES-1 expression in chondrocytes. Indeed, cell culture experiments demonstrated that IL-1ß induced mPGES-1 protein expression in a dose-dependent manner. Time course analysis showed that mPGES-1 protein started to increase 6 h post-stimulation with IL-1ß and remained elevated even 48 h after IL-1ß stimulation. This is in contrast to other IL-1ß-induced genes in chondrocytes, in which expression is rapidly induced (2–3 h), reaching a maximum at 8 h, and then gradually decreases to reach basal levels at 24–36 h. These differences in the kinetics of induction suggest that the mechanisms controlling the expression of these genes are not identical.

Upregulation of mPGES-1 expression by IL-1ß occurred, at least in part, at the transcriptional level, as determined by real-time quantitative RT-PCR and transient transfection experiments. With regard to the mechanism by which IL-1ß induces mPGES-1 transcription, it is known that the human mPGES-1 promoter contains several potential transcription factor-binding sites, including 2 GC boxes, 2 Barbie boxes, and an aryl hydrocarbon response element[31]. Naraba, *et al*[32] showed that the binding of Egr-1, an inducible transciption factor, to the proximal GC box plays an essential role in the induction of mPGES-1 in macrophages and osteoblastic cells. This is consistent with other reports that Egr-1 is important for mPGES-1 transcription in human colonocytes[33] and synovial fibroblasts[34]. Although we have not investigated Egr-1, it seems likely that this transcription factor may also play an important role in induced mPGES-1 gene expression in chondrocytes.

Proinflammatory cytokines TNF-α and IL-17 have also been implicated in the pathogenesis of arthritis. These medi-

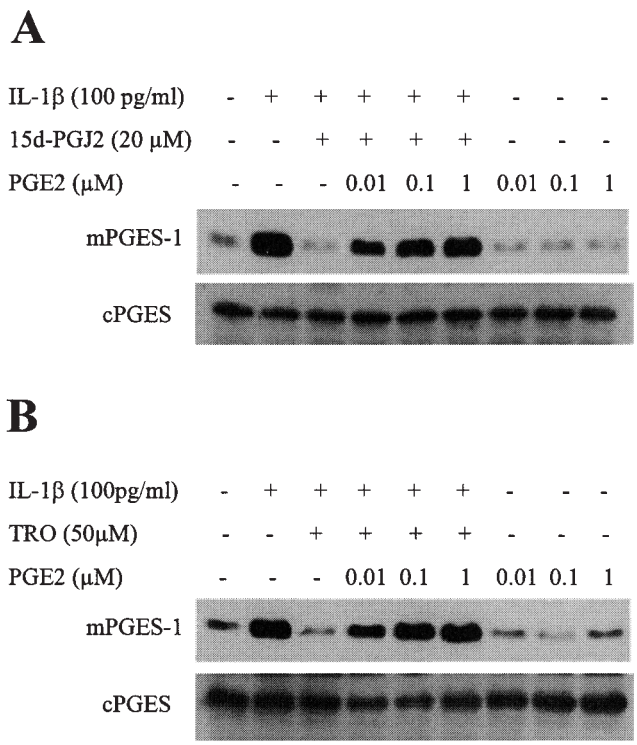


Figure 7. PPARγ ligand-inhibited IL-1ß-induced mPGES-1 expression is alleviated by PGE_2. Chondrocytes were pretreated with increasing concentrations of PGE_2 for 30 min. The cells were then treated with or without IL-1ß (100 pg/ml) for 24 h in the absence or presence of 20 μM 15d-PGJ_2 (A) or 50 μM troglitazone (B). Cell lysates were prepared and analyzed for mPGES-1 protein by Western blotting. In the lower panels, the blots were stripped and reprobed with a specific anti-cPGES antibody. The blots are representative of similar results obtained from 4 independent experiments.

ators are present at elevated levels in articular joint tissues and are believed to induce their effects through enhancing production of a number of inflammatory and catabolic factors[18,19,35,36]. We found that TNF-α and IL-17, like IL-1ß, also induced mPGES-1 expression. At optimal concentrations, the effect of IL-17 or TNF-α on mPGES-1 expression and PGE_2 production was less potent than that of IL-1ß. Recently, numerous studies have described synergistic or additive effects between IL-1ß, TNF-α, and IL-17 in many systems. Katz, *et al*[20] demonstrated in human OA synovial fibroblasts that the combination of IL-17 and TNF-α synergistically stimulated production of IL-1, IL-6, and IL-8. Chabaud, *et al*[21] found that combinations of IL-1ß, TNF-α, and IL-17 had a synergistic effect on production of macrophage inflammatory protein-3α by RA synovial fibroblasts. Berenbaum, *et al*[24] showed that IL-1ß and TNF-α synergize to induce PGE_2 production and COX-2 expression in rabbit articular chondrocytes. Synergy between IL-1ß, TNF-α, and IL-17 on nitric oxide (NO) and PGE_2 production by explants of human OA knee menisci was demonstrated by LeGrand, *et al*[22]. Here, we extend these findings by showing that combinations of IL-1ß, TNF-α, and IL-17 were synergistic on induction of mPGES-1 protein expression. Moreover, the effect of the combination of 3 cytokines was stronger than that of each of the 2 cytokines, suggesting that the combination of cytokines may be of importance in increased expression of mPGES-1. As expected, the level of cPGES expression was not altered by these treatments. mPGES-2 protein was also present in cultured chondrocytes, but its expression did not change with any of the treatments used in our studies (data not shown). This is consistent with other reports showing that expression of mPGES-2 is not affected by proinflammatory stimuli in several cell types, including chondrocytes[27,37].

Recently, numerous studies have shown that PPARγ ligands inhibit expression of several genes involved in the pathogenesis of arthritis. For example, PPARγ ligands prevent expression of IL-1, IL-6 and TNF-α in activated monocytes/macrophages, of collagenase-1 in synovial fibroblasts, and of collagenase-3 and inducible NO synthase in chondrocytes[25,38]. PPARγ ligands were also shown to inhibit induction of PGE_2 production in a number of experimental systems[34,39,40]. In our study, we showed that the PPARγ ligands 15d-PGJ_2 and troglitazone, but not the PPARα ligand Wy14643, repressed IL-1ß-induced mPGES-1 expression. Interestingly, treatment with PGE_2 restored expression of mPGES-1. However, PGE_2 had no significant effect on unstimulated mPGES-1 expression, indicating that additional signals are provided by IL-1ß stimulation, which PGE_2 alone cannot provide. These data also suggest that upregulation of mPGES-1 is dependent, at least in part, on PGE_2 production. This suggestion is supported by findings that inhibition of IL-1ß-induced mPGES expression by NSAID is restored by exogenous AA and PGE_2[41,42].

In addition to its proinflammatory effects, elevated biosynthesis of PGE_2 has been associated with erosion of cartilage and juxtaarticular bone. PGE_2 can contribute to joint tissue damage by inhibiting collagen and proteoglycan synthesis, promoting the production of matrix metalloproteases and suppressing synthesis of tissue inhibitor of metalloproteases[5,43]. In addition, PGE_2 triggers osteoclastic bone resorption[5]. The upregulation of mPGES-1 in cartilage from OA patients suggests its involvement in local increased PGE_2 production and tissue destruction. This is supported by findings that degradation of cartilage and bone were reduced in mPGES-1-deficient mice[13,44].

Our data show that expression of mPGES-1 is upregulated in OA cartilage. The proinflammatory cytokines IL-1ß, TNF-α, and IL-17 may be responsible for this upregulation. Combined with results from previous studies showing a critical role of mPGES-1 in the synthesis of PGE_2 and the pathogenesis of arthritis[11-13,44], these data suggest that mPGES-1 constitutes a novel therapeutic target in the treatment of arthritis and possibly other diseases in which increased production of PGE_2 is implicated.

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