

Feedback Control of Cyclooxygenase-2 Expression by Prostaglandin E₂ in Rheumatoid Synoviocytes

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ABSTRACT

Objective: The role of prostaglandin E₂ (PGE₂) in the etiopathogenesis of immune and inflammatory diseases has become the subject of recent debate. To determine the role of PGE₂ in rheumatoid arthritis (RA), we tested the effect of exogenous PGE₂ on the production of cyclooxygenase-2 (COX-2) by rheumatoid synoviocytes. **Methods:** Fibroblast-like synoviocytes (FLS) were prepared from the synovial tissues of RA patients, and cultured in the presence of PGE₂. The COX-2 mRNA and protein expression levels were determined by RT-PCR and Western blot analysis, respectively. The PGE₂ receptor subtypes in the FLS were analyzed by RT-PCR. Electrophoretic mobility shift assay (EMSA) was used to measure the NF- κ B binding activity for COX-2 transcription. The in vivo effect of PGE₂ on the development of arthritis was also tested in collagen induced arthritis (CIA) animals. **Results:** PGE₂ (10^{-11} to 10^{-5} M) dose-dependently inhibited the expression of COX-2 mRNA and the COX-2 protein stimulated with IL-1 β , but not COX-1 mRNA. NS-398, a selective COX-2 inhibitor, displayed an additive effect on PGE₂-induced COX-2 downregulation. The FLS predominantly expressed the PGE₂ receptor (EP) 2 and EP4, which mediated the COX-2 suppression by PGE₂. Treatment with anti-IL-10 monoclonal antibodies partially reversed the PGE₂-induced suppression of COX-2 mRNA, suggesting that IL-10 may be involved in modulating COX-2 by PGE₂. Experiments using an inducer and an inhibitor of cyclic AMP (cAMP) suggest that cAMP is the major intracellular signal that mediates the regulatory effect of PGE₂ on COX-2 expression. EMSA revealed that PGE₂ inhibited the binding of NF- κ B in the COX-2 promoter via a cAMP dependent pathway. In addition, a subcutaneous injection of PGE₂ twice daily for 2 weeks significantly reduced the incidence and severity of CIA as well as the production of IgG antibodies to type II collagen. **Conclusion:** Our data suggest that overproduced PGE₂ in the RA joints may function as an autocrine regulator of its own synthesis by inhibiting COX-2 production and may, in part, play an anti-inflammatory role in the arthritic joints. (*Immune Network* 2003;3(3):201-210)

Key Words: Prostaglandin E₂, synoviocytes, COX-2, cAMP, NF- κ B

Introduction

Prostaglandin E₂ (PGE₂), an arachidonic acid metabolite, exerts diverse effects on the immune response and the biological outcome in a variety of inflammatory diseases including rheumatoid arthritis (RA). It was initially believed to be a pro-inflammatory mediator causing vasodilation, hyperalgesia, and

fever (1,2), but has progressively been recognized for its immuno-regulatory and anti-inflammatory activities (3-6). PGE₂ dose-dependently inhibits IL-2 and IFN- γ production in stimulated human peripheral blood lymphocytes, although IL-5 production is increased (3). These divergent actions of PGE₂ on Th1 and Th2 cytokines were reproduced in other experiments reported by Katamura et al (4). They showed that naive CD4⁺ T cells from cord blood lost their ability to produce IFN- γ and IL-2 at the mRNA level after being treated with PGE₂. PGE₂ also strongly inhibited the LPS-induced production of IL-12, a critical cytokine of the Th1 response (5). In contrast, PGE₂ induced IL-10 approximately two fold in same condition. Wu et al also reported that

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the surface expression of IL-12R β 1, IL-12R β 2 mRNA, and the receptor binding of IL-12 were diminished in PBMC after a treatment with PGE2 (6). This inhibitory effect of PGE2 is probably due to its cyclic AMP (cAMP) inducing capacity because other cAMP inducers or cAMP analogues mimic its action (4-6).

The regulatory function of PGE2 on the target cells is exerted through receptor-coupling events. There are at least four subtypes of G-protein-coupled cell surface receptors, termed EP1, EP2, EP3, and EP4 receptors that bind (7,8). However, the consequence of PGE2 binding differs according to the receptor subtypes. For example, the EP3 receptor couples to Gi and inhibits adenylate cyclase. In contrast, EP2 or EP4 receptor couples to Gs and activates adenylate cyclase, resulting in an elevation of intracellular cAMP (9,10). Elevated cAMP subsequently induces the transcriptional activation of anti-inflammatory cytokines such as IL-10 via the activation of the cAMP responsive element-binding proteins (CREB) (11,12), whereas inhibits NF- κ B-mediated transcription (13,14). It has been documented that PGE2 can block the DNA binding activity of NF- κ B to the IL-2 transcriptional starting site (15).

The rate limiting step of PGE2 synthesis is cyclooxygenase (COX) expression. This enzyme catalyzes the conversion of arachidonic acid to PGH2, a precursor of the biologically active prostaglandins. Two COX isoforms have been described, COX-1 and COX-2. COX-1 is a constitutive isoform that is expressed in most human cells and tissues (16). COX-2 is an inducible form of the COX enzyme, which is induced by stimulation by cytokines, mitogens, and bacterial endotoxins (16-18). In particular, the influence of multiple cytokines on COX-2 expression has been reported in many *in vitro* studies. Proinflammatory cytokines including TNF- α (19,20), IL-1 α (20), and IFN- γ (21) have been demonstrated to induce COX-2 expression, whereas anti-inflammatory cytokines such as IL-4 (22) and IL-10 (23) have been reported to inhibit COX-2 induction. Therefore, it is possible that COX-2 expression may be finely regulated by a number of cytokines within the RA joints. However, it is unclear whether PGE2, which is highly expressed in RA joints (24,25), can suppress COX-2 expression in RA as effective as it inhibit the production of Th1 and proinflammatory cytokines.

Here, it is shown that exogenous PGE2 strongly inhibits COX-2 expression in fibroblasts-like synovocytes (FLS) of RA via the EP2 and EP4 receptors. The inhibition of COX-2 transcription appears to be affected by an increase in IL-10, at least in part. The effects of PGE2, including the reduced NF- κ B binding activity to a COX-2 promoter, can be mim-

icked by a cAMP inducer but completely abrogated by a cAMP inhibitor. Moreover, PGE injection reduces the incidence and severity of collagen (CII)-induced arthritis (CIA). Collectively, these results suggest that PGE2 strongly inhibits NF- κ B-mediated activation of COX-2 gene transcription in FLS via a cAMP-dependent pathway and has the potential to suppress RA inflammation.

Materials and Methods

Reagents and antibodies. PGE2 (Dinoprostone), 3-isobutyl-1-methylxanthine (IBMX), and 2'-3'-dideoxyadenosine (DDA) were purchased from Sigma (St. Louis, MO). The LPS was also obtained from Sigma. NS-398, a COX-2 inhibitor, was obtained from Calbiochem (La Jolla, CA). The EP2/EP4 agonist, 11-deoxy-PGE2, and the EP1/EP3 agonist, sulprostone, were purchased from Cayman Chemical (Ann Arbor, MI). The recombinant IL-1 β and IL-10 were purchased from R & D systems (Minneapolis, MN), respectively. [γ - 32 P] ATP was purchased from Amersham Pharmacia (Uppsala, Sweden). Reagents used for the culture contained <200 pg/ml of the endotoxin, as determined by a *Limulus* amoebocyte cell lysate assay.

Isolation of synovocytes. The FLS cell lines were prepared from the synovial tissues of 6 RA patients undergoing a total joint replacement surgery according to the procedure described previously (26). The mean age of the patients (5 females and 1 male) was 52.6 years. The mean disease duration was 81.4 months. Five out of six patients had a positive rheumatoid factor. All had erosions as indicated by hand X-rays. The cells between the forth and the eight passages were used, during which time they comprised a FLS homogenous population (<2.5% CD 14, <1% CD3, and <1% CD19 positive by flow cytometry analysis).

Culture conditions. The FLS cells were seeded in 24 well plates at a concentration of 6×10^4 cells per well in 1 ml DMEM/5% FCS, and incubated at 37°C for 24 h in the presence of varying PGE2 concentrations, ranging from 10^{-11} to 10^{-5} M. To induce COX-2 synthesis in FLS, 10 ng/ml of the IL-1 β was added to the wells at the onset of culturing with PGE2. In some experiments, NS-398, a selective COX-2 inhibitor, was simultaneously treated to the wells in the presence of PGE2 and IL-1 β . To investigate the effect of endogenous IL-10 on COX-2 expression, IL-10 was neutralized by adding monoclonal antibodies (mAbs) to IL-10. Various concentrations of a cAMP inducer, IBMX, were used to determine whether they could mimic the effect of PGE2. A cAMP inhibitor, DDA, was used to block cAMP accumulation. Both IBMX and DDA were added to each well at the onset of the culture. After 24 h of incubation, unless otherwise stated, the cell-free me-

dium was collected and stored at -20°C until assayed. All cultures were set up in either triplicate or quadruplicate.

RNA isolation and RT-PCR for COX-2 mRNA. The FLS were incubated with various concentrations of IL-1 β . After 6 h of incubation, which was optimal for COX-2 induction, the mRNA was extracted using RNAzol B according to the manufacturer's instruction (Biotex Laboratories, Houston, TX). The reverse transcription of 5 μg total mRNA was carried out at 42°C using the SuperscriptTM reverse transcription system (Life Technologies). PCR amplification of the cDNA aliquots was performed by adding 2.5 mM dNTPs, 2.5 U Taq DNA polymerase (TaKaRa SHUZO Co., Shiga, Japan) and 0.25 μM each of the sense and antisense primers. The reaction was done in a PCR buffer (1.5 mM MgCl_2 , 50 mM KCl, 10 mM Tris HCl, pH 8.3) in a total volume of 25 μl . The following sense and antisense primers for each molecules were used (all written in 5' \rightarrow 3' direction): COX-2 sense GCAGTTGTTCAGACAAGCA, COX-2 antisense C-AGGATACAGCTCCACAGCA; GAPDH sense CGATGCTGGGCGTGAGTAC, GAPDH antisense CGTTCAGTCCA-GGGATGACC. The reactions were processed in a DNA thermal cycler (Perkin-Elmer, Foster City, CA) through cycles of 5 min denaturation at 94°C , 1 min annealing at 55°C for GAPDH and 45 seconds at 55°C for COX-2, and 30 seconds elongation at 72°C . The PCR rounds were repeated for 25 cycles for GAPDH and 27 cycles for COX-2, which were determined to fall within the exponential phase of amplification for each molecule. PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. The mRNA expression level was presented as a ratio of the cytokine PCR product over the GAPDH product.

Western blot analysis for COX-2 protein. The FLS (5×10^5 cells) were incubated for 12 h in the presence or absence of IL-1 β . After incubation, the cells were harvested and lysed in 150 μl of solubilization buffer (1% Tween 20, 10 mmol/L phenylmethylsulfonyl fluoride, and 50 mmol/L Tris-HCl, pH 8.0). The protein extracts (25 μg) were then separated on a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with PBS containing 5% skim milk and 0.1% Tween 20, and then incubated with 0.25 $\mu\text{g}/\text{ml}$ of rabbit anti-human COX-2 polyclonal Abs in the blocking buffer at 25°C for 2 h. The membrane was subsequently incubated with peroxidase-conjugate anti-rabbit IgG (1 : 1000 dilution) and analyzed using an Amersham enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). Fuji X-omat AR film (Fuji Photo Film Co, Tokyo, Japan) with

cassette closure times of 5 to 10 minutes was used to obtain adequate exposure and to visualize the bands.

RT-PCR for subtypes of prostaglandin E2 receptors. The total RNA was extracted from synoviocytes of RA patients using the TRIzol reagent according to the manufacturer's instruction (Life Technologies). The RT-PCR was performed with 2 μg of RNA in the first strand synthesis in a total volume of 20 μl containing 80 pmol of the random hexamer primers (TaKaRa SHUZO Co.), 10 mM DTT solution, 0.5 mM dNTPs, 40 U of RNase inhibitor (TaKaRa SHUZO Co.), and 200 U of Superscript II reverse transcriptase (Invitrogen, Groningen, Netherland). One microliter of the product was for PCR in a total volume of 25 μl with 10 pmol each of the appropriate forward and reverse primers, 200 μM dNTPs, and 0.5 U DNA Taq Polymerase (TaKaRa SHUZO Co.) in 10 \times buffer. The PCR amplification was carried out in a PCR thermal cycler (Perkin-Elmer Cetus) with an initial denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation for 30 seconds at 94°C , annealing for 30 seconds at 56°C , and extension time of 30 seconds at 72°C , with a final extension time of 10 minutes at 72°C . The upstream and downstream primers (5' \rightarrow 3') were TTGTCCGGTATCATGGTGGTG and GGCCTCTGGTTGTGCTT-AGA for EP1, CAAGCCTGCTGTCTTGTGAT and TCCTCCCCCAGAAATAGGAT for EP2, AACTGGGGCAACCTTTTCTT and TTTCTGCTTCTCCGTGTGTG for EP3, and TGTGAACCCCATCC-TAGACC and GCACACCTGGAAGCAAATTC for EP4. The PCR products were analyzed on a 1.5% agarose gel.

EMSA (Electrophoresis Mobility Shift Assay). The FLS nuclear extract was prepared as previously described (27). For the induction of NF- κB activity, the cells were pretreated with 10 ng/ml of IL-1 β for 1 h in the presence or absence of PGE2. A double-stranded oligonucleotide probe containing the NF- κB recognition site (underlined) of human COX-2 promoter (5'-GGAGAGGGGATTCCTGCGCC-3') was generated by a 5'-end labeling of the sense strand with [γ - ^{32}P] dATP using T4 polynucleotide kinase (TaKaRa SHUZO Co.), and purified by NucTrap columns (Stratagene, La Jolla, CA). The NF- κB binding reactions were performed with 2 μg of the nuclear extract in 10 μl of the binding buffer containing 400,000 cpm of the labeled oligonucleotide for 30 min at room temperature. A 100 \times excess of the unlabeled oligonucleotide was used as a competitor. The DNA-protein complex was analyzed on a 5% polyacrylamide gel that was electrophoresed in a TBE buffer. The gels were then dried and exposed to BioMax-MR film (Eastman Kodak Co, Rochester, NY) at -70°C at 24 h.

The supershift assay was performed to verify the identity of the bound factors using specific antibodies to p65, p50, and c-Rel proteins. Briefly, 100× of Abs against p65, p50 and c-rel (Santa Cruz Laboratory, Santa Cruz CA) were added to the binding reaction prior to addition of the probe, and then incubated on ice for 30 min.

Determination of *in vivo* effect of PGE2 in collagen-induced arthritis. Male DBA/1 mice, which were obtained from Jackson Laboratories (Bar Harbor, ME) were maintained in groups of three to five in polycarbonate cages and fed standard mouse chow (Ralston Purina, St. Louis, MO) and water ad libitum. The environment was made specifically pathogen-free for the mice. Neonatal mice were obtained by breeding the mice in our facility. The mice were immunized with native CII, a generous gift from Dr. Andrew H Kang (University of Tennessee, Memphis, TN), at 8 to 12 weeks of age. For the injection, CII was dissolved in 0.1 N acetic acid at 2 mg/ml, and emulsified (1 : 1 ratio) with complete Freund's adjuvant (CFA) at 4°C (28). The mice received 0.1 ml of the emulsion containing 100µg of CII in the base of the tail as a primary immunization. Booster injections were given into the footpad with 50µg of CII similarly dissolved and emulsified 1 : 1 with CFA 14 days after the primary immunization. From 3 weeks after the primary immunization, 10 to 20µg of PGE2 dissolved in PBS was injected subcutaneously in the CIA animals twice daily during 2 weeks. The control mice received PBS alone. The incidence and severity of arthritis in the two groups of mice were determined using a visual inspection. The mice were observed two to three times a week for the onset, duration, and severity of joint inflammation over a period of 10 weeks after the primary immunization. Each limb was assessed on a 0- to 4-point scale, as described earlier (28). The hindfoot that received the booster immunization was excluded from the evaluation. Therefore, the maximum arthritis score possible was 12. The mean arthritic index and incidence of arthritis (%) were used for data comparison among the experimental groups.

Assay for IgG antibodies to type II collagen. Sera were collected from each group of mice on day 35 after the primary immunization and stored at -20°C until the assay. The IgG anti-CII levels in the sera were determined by a commercial enzyme-linked immunosorbent assays kit (Chondrex, Redmond, WA), as previously described (28). The optical density of the standard serum, which was serially diluted 2-fold, is expressed as 100, 50, 25, 12.5, 6.25, 3.125 arbitrary units, respectively. The relationship of the optical density measured in the standard serum diluted serially and arbitrary units showed a good linear correlation in

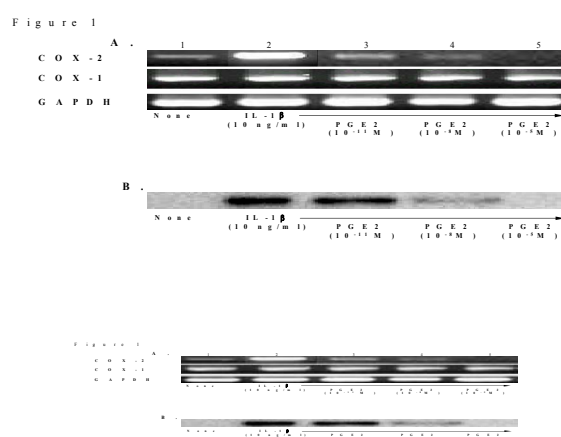


Figure 1. Repression of IL-1 β -induced cyclooxygenase 2 expression by prostaglandin E2 (PGE2). (A) Messenger RNA expression of the cyclooxygenases in the fibroblasts-like synoviocytes (FLS). The FLS were incubated with PGE2 in the presence or absence of IL-1 β (10 ng/ml). After 6 h of incubation, which is optimal for COX-2, the FLS were analyzed for COX-2 mRNA expression by semi-quantitative RT-PCR. COX-2 mRNA expression after treatment without (lane 1) or with IL-1 β (lane 2), IL-1 β plus 10⁻¹¹ M PGE2 (lane 3), IL-1 β plus 10⁻⁸ M PGE2 (lane 4), IL-1 β plus 10⁻⁵ M PGE2 (lane 5). COX-1 amplification was used as a negative control. The mRNA levels are corrected for the levels of the GAPDH mRNA signal. Representative data from three independent experiments is shown. (B) Western blot analysis of the COX-2 protein. FLS were incubated for 12 hr with various PGE2 concentrations in the presence of IL-1 β (10 ng/ml). The whole cell lysate of the FLS (5×10⁵ cells) was subjected to immunoblot analysis using polyclonal antibodies against the purified COX-2 proteins. The other culture conditions were similar to those used for RT-PCR.

all determinations ($r > 0.98$, data not shown). The IgG anti-CII concentrations in the sera diluted 1 : 4,000 are presented as relative values (arbitrary units) compared to the optical density of the standard sera.

Statistical analysis. The data is expressed as a mean \pm the standard deviation (SD). Statistical analysis was performed using the Student's *t*-test for matched pairs. Differences with a confidence level of 95% or higher were considered to be statistically significant ($P < 0.05$).

Results

PGE2 inhibits IL-1-induced COX-2 expression in rheumatoid synoviocytes. As shown in Fig. 1A, the unstimulated FLS expressed COX-2 mRNA slightly, which was strongly upregulated by 10 ng/ml of IL-1 β . Incubation of the cells with PGE2 (10⁻¹¹ to 10⁻⁵ M) resulted in a dose-dependent decrease in IL-1 β induced COX-2 mRNA expression to their spontaneous levels. The increase in the COX-2 protein levels induced by IL-1 β was also dose-dependently inhibi-

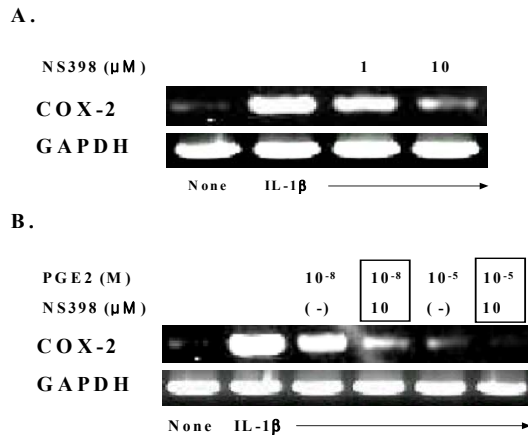


Figure 2. Effect of NS-398 on PGE2-mediated COX-2 regulation. (A) Dose dependent decrease in the COX-2 mRNA by NS-398. NS-398 (1 or 10 μM) was added to fibroblasts-like synoviocytes (FLS) in the presence or absence of IL-1 β (10 ng/ml). The COX-2 mRNA expression level was determined by semi-quantitative RT-PCR. (B) Additive effect of NS-398 on PGE2-mediated COX-2 down-regulation. Ten μM of NS-398 was simultaneously added to the FLS with 10⁻⁸ M or 10⁻⁵ M of PGE2 in the presence of IL-1 β . The boxes indicate the co-treatment of NS-398 with PGE2.

ted by the treatment with PGE2 (Fig. 1B). However, the COX-1 mRNA levels were not changed by either IL-1 β or PGE2. These results suggest that PGE2 could regulate its own synthesis by blocking COX-2 expression.

It is well known that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the COX-2 production, a key enzyme for the endogenous PGE2 function. Moreover, NSAIDs such as aspirin and salicylic acid repress the NF- κ B-responsive genes in monocytes (29). Assays performed in the presence of a COX-2 specific inhibitor NS-398 (1 μM and 10 μM), showed that this agent dose-dependently decreased COX-2 mRNA expression (Fig. 2A). Moreover, NS-398 potentiated the downregulation of COX-2 mRNA when added together with PGE2 (Fig. 2B). This suggests that the blockade of endogenous PGE2 by NSAIDs did not attenuate the downregulatory effect of exogenous PGE2 on COX-2 expression.

EP2 and EP4 receptors are involved in COX-2 inhibition in response to exogenous PGE2. PGE2 functions through the receptor-coupling events (7-10). To investigate which type of EP receptors predominantly mediates PGE2-induced COX-2 suppression, we examined the

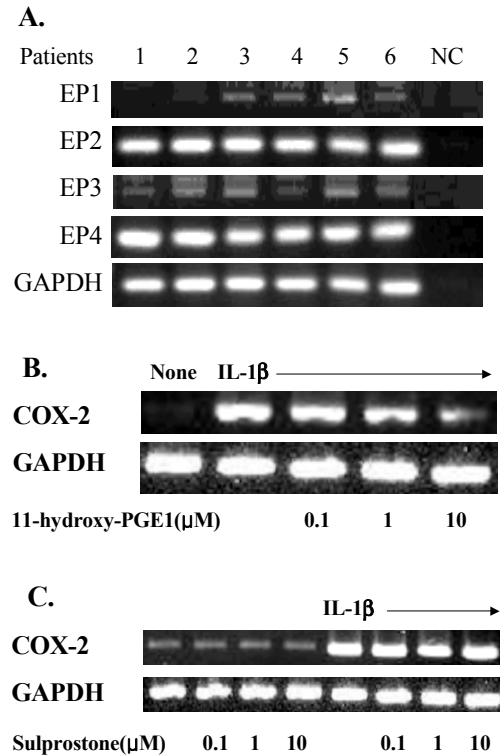


Figure 3. Involvement of prostaglandin receptor (EP) 2 and EP4 subtypes in COX-2 inhibition in response to exogenous PGE2 (A) Analysis of the EP subtypes in the synovial fibroblasts. The expression of the EP subtypes (EP1, EP2, EP3, and EP4) was determined by RT-PCR in the FLS from six different patients with rheumatoid arthritis. GAPDH amplification was used as internal control. NC indicates the negative control without cDNA. (B and C) Effect of hydroxy-PGE1 (EP2/EP4 agonist) and sulprostone (EP1/EP3 agonist) on the IL-1 β -induced COX-2 expression. Various concentrations of hydroxy-PGE1 and sulprostone, ranging from 0.1 to 1 μM , were added to the FLS stimulated with 10 ng/ml of IL-1 β . COX-2 mRNA expression was determined by RT-PCR. Representative data from three independent experiments is shown.

mRNA expression of EP four subtypes (EP1, EP2, EP3, EP4) in the FLS obtained from 6 RA patients. In all 6 RA patients, FLS strongly expressed two subtypes of the PGE2 receptor, EP2 and EP4, as revealed by RT-PCR (Fig. 3A). EP1 and EP3 were also detected in 4 to 5 FLS out of the 6 cell lines, but the band densities of the PCR products were faint compared to the EP2 and EP4 under similar PCR conditions. Moreover, 1-hydroxy-PGE1 dose-dependently inhibited the COX-2 expression stimulated with IL-1 β when the FLS were treated with 1-hydroxy-PGE1, which has been shown to have high affinity for EP2 and EP4. In contrast, sulprostone,

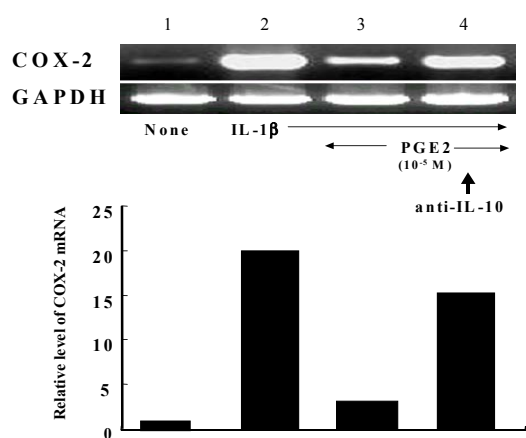


Figure 4. IL-10 dependency of PGE2-mediated COX-2 suppression. FLS were cultured with 10^{-5} M of PGE2 in the presence or absence of neutralizing antibodies (Abs) to IL-10. COX-2 mRNA expression after treatment without (lane 1) or with IL-1β (lane 2), IL-1β plus 10^{-5} M of PGE2 (lane 3), and IL-1β plus 10^{-5} M of PGE2 in the presence of 10 μg/ml of anti-IL-10 (lane 4). The levels of mRNA are expressed as the fold increase relative to the mRNA from the unstimulated control cells, and corrected for the levels of the GAPDH mRNA signal. The data represents the results from one of three similar experiments.

an EP1/EP3 agonist, had no significant effect on the basal and IL-1β-induced COX-2 expression levels (Fig. 3B). These results suggest that the EP2 and EP4 receptor may be predominantly involved in COX-2 inhibition by exogenous PGE2.

Suppression of COX-2 by PGE2 was partially mediated by the increase of IL-10. IL-10 is a central regulator of COX-2 expression (30). We demonstrated previously that PGE2 increased IL-10 production from FLS. Consequently, we tested whether or not increased IL-10 by PGE2 acted as an intermediate in COX-2 suppression. As shown in Fig. 4, incubation of FLS with neutralizing anti-IL-10 mAbs in the presence of 10^{-5} M of PGE2 resulted in the partial recovery of COX-2 mRNA stimulated by IL-1β. In this experiment, the equivalent concentration of the isotype control Abs (IgG1; R & D systems) did not show any significant effect (data not shown). These observations indicate that the suppression of COX-2 by PGE2 is partially dependent on the enhancement of IL-10 production.

PGE2 inhibits NF-κB-mediated COX-2 transcription via a cAMP dependent pathway. The activation of adenylate cyclase and the resultant accumulation of cAMP are

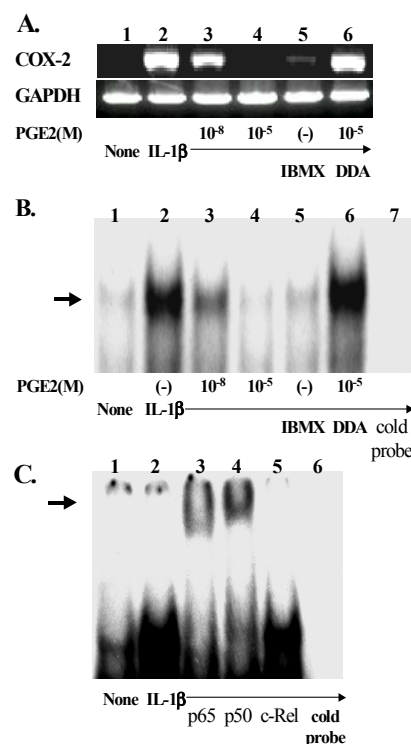


Figure 5. PGE2 inhibits NF-κB-mediated COX-2 transcription via a cAMP dependent pathway. The IL-1β-stimulated FLS were incubated with the cyclic AMP (cAMP) agonists, 3-isobutyl-1-methylxanthine (IBMX) or c-AMP antagonist, 2'-3'-dideoxyadenosine (DDA) with in the presence or absence of PGE2. (A) cAMP dependency of COX-2 mRNA suppression by PGE2. COX-2mRNA expression after treatment without (lane 1) or with IL-1β (lane 2), IL-1β plus 10^{-8} M PGE2 (lane 3), IL-1β plus 10^{-5} M PGE2 (lane 4), IL-1β plus IBMX 500 μM (lane 5), and IL-1β plus 10^{-5} M PGE2 in the presence of DDA 500 μM (lane 6). (B) Effect of PGE2 and the cAMP inducer on NF-κB complex in the nuclear extracts of FLS. The cells were pretreated with 10 ng/ml of IL-1β for the induction of NF-κB activity for 1 h prior to the analysis. The NF-κB binding activity in the nuclear extract was determined by EMSA, as described in Material and Methods. The abrogation of NF-κB activity by an excess of unlabeled oligonucleotide in the IL-1β-stimulated cells (lane 7), the NF-κB activity in the absence (lane 1) or presence IL-1β (lane 2), IL-1β plus 10^{-8} M PGE2 (lane 3), IL-1β plus 10^{-5} M PGE2 (lane 4), IL-1β plus IBMX 500 μM (lane 5), and IL-1β plus 10^{-5} M PGE2 in the presence of DDA 500 μM (lane 6). The arrow on the left denotes the labeled oligonucleotide band shifted upon NF-κB binding. A representative result from three independent experiments using the cell lines of different RA patients is shown. (C) Supershift assay of the NF-κB site using antibodies against p65 (lane 3), p50 (lane 4), and c-Rel (lane 5). The cells in all lanes, except lane 1 (medium alone), were pre-stimulated with IL-1β. The arrow on the left denotes the labeled oligonucleotide band shifted upon NF-κB binding.

downstream pathways activated by PGE2 receptor signaling (4-6). The agents known to enhance cAMP were analyzed to determine if they could mimic

PGE2 action on COX-2 expression. IBMX, a phosphodiesterase inhibitor, suppressed the expression of COX-2 mRNA stimulated by IL-1 β , whereas DDA, an adenylate cyclase inhibitor, completely abrogated the effect of PGE2 (Fig. 5A), suggesting that the COX-2 regulation by PGE2 is mainly mediated via a cAMP-dependent pathway.

IL-1 β has been associated with the induction of the nuclear factor- κ B (NF- κ B) and increased COX-2 transcription (31), on the basis of the existence of two putative NF- κ B-binding motifs on human COX-2 gene (32). We tested if the accumulation of cAMP by PGE2 is related to the down-regulation of COX-2 promoter activation, by a mobility shift assay of the NF- κ B site. Fig. 5B shows that when compared to unstimulated cells (lane 1), the incubation of the FLS with IL-1 β (10 ng/ml) strongly induced the DNA-binding activity of NF- κ B (lane 2). The shifting of the radiolabeled NF- κ B element was

chased by a competition with an excess amount of the unlabeled oligonucleotide (lane 7). The addition of PGE2 to the IL-1 β -stimulated FLS cultures resulted in a dose-dependent reduction in NF- κ B binding (lane 3 and 4). Treatment with IBMX has a similar result to that of PGE2 10^{-5} M (lane 5), whereas DDA completely restored the NF- κ B activity (lane 6). These results suggest that PGE2 inhibits COX-2 mRNA production via the elevation of cAMP, which in turn blocks NF- κ B-signaling through the COX-2 promoter. A supershift assay using antibodies to p65, p50, and *c-rel* was performed to verify the identity of binding NF- κ B isoforms (Fig. 5C). It appears that the factors binding to the oligonucleotide include p65 and p50, but not *c-rel*.

Suppression of collagen-induced arthritis by PGE2. Finally, we investigated the *in vivo* effect of exogenous PGE2 on the progression of arthritis. As shown in Fig. 6, a subcutaneous injection of PGE2 (10 to 20 μ g) twice

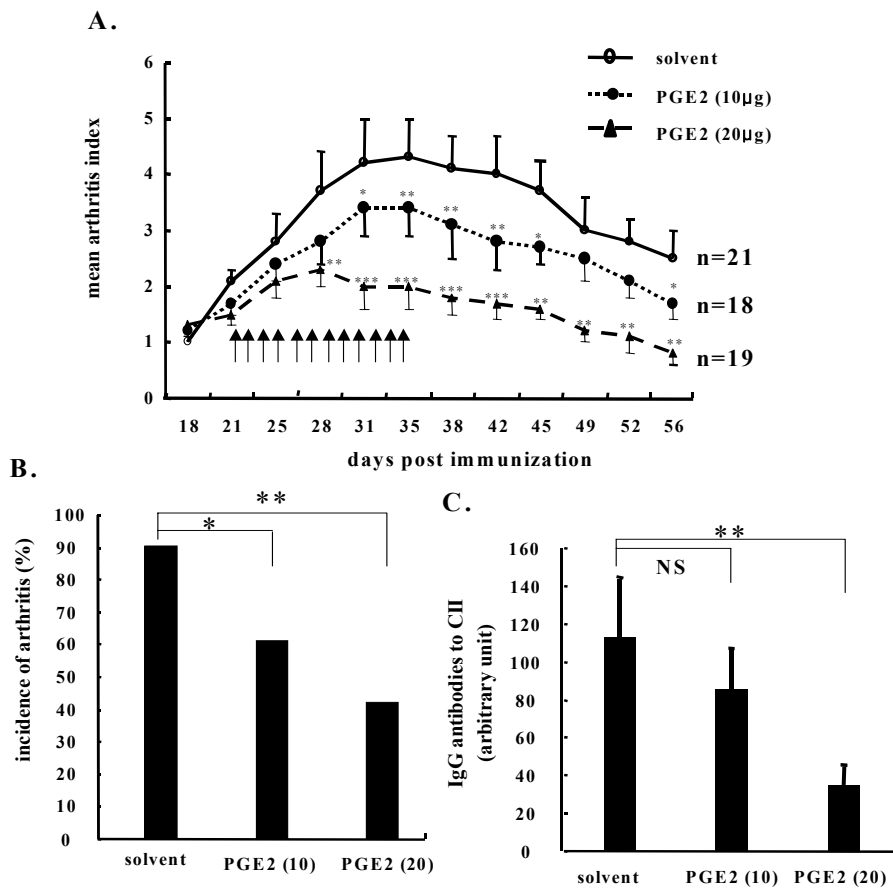


Figure 6. Suppression of collagen induced arthritis (CIA) by PGE2. From 3 weeks after the primary immunization, 10 (closed circles) or 20 μ g (closed triangle) of PGE2 dissolved in PBS was injected in the CIA animals twice daily for 2 weeks. The control mice (open circles) were received PBS alone. The severity and incidence of arthritis were determined in the three groups of mice by a visual inspection. The arrows denote a subcutaneous injection with PGE2 or the solvent. (A) Decrease in the arthritis index in the PGE2-injected mice during the course of CIA. The data is presented as the mean (\pm SD) values. (B) Reduction in the incidence of arthritis in the PGE2-treated mice, as determined 5 weeks after immunization. (C) The levels of circulating IgG antibodies to CII in PGE2 versus the solvent-treated mice, as determined 5 weeks after primary immunization. Data are presented as the mean (\pm SD) titers of the circulating IgG anti-CII antibodies. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus solvent alone.

daily for 2 weeks reduced the incidence and severity of CIA in a dose-dependent manner (Fig. 6A and 6B). In addition, the serum levels of the IgG antibodies to CII were significantly lower in the mice treated with PGE₂ than in the mice treated with PBS alone, as determined 5 weeks after a primary immunization (Fig. 6C). This clearly shows that exogenous PGE₂ has strong anti-inflammatory activity *in vivo*. On histological examination of the joints, it was also found that the paws of the PGE₂-treated (20 µg twice) mice exhibited a lower degree of inflammation, cartilage destruction, and synovial proliferation compared to the PBS-treated mice, as determined on day 35 after immunization (data not shown).

Discussion

Enhanced COX-2 production has been associated with the pathogenesis of RA (33-36). In RA synovium, both COX-1 and -2 are highly expressed, with COX-2 expression being elevated in relation to the degree of the inflammation in the synovial tissue (33,34). Blocking COX-2 activity with a selective inhibitor prevented the progression of arthritis and the production of the pro-inflammatory cytokines such as TNF-α and IL-6, which are key mediators of rheumatoid inflammation (35). Recently, Myers et al. reported that CIA was prevented in genetically COX-2 depleted mice whereas COX-1 depleted mice could not be protected from developing CIA (36), thereby demonstrating the crucial role of COX-2 in RA inflammation.

We have previously demonstrated that PGE₂ strongly suppresses IL-15 production, whereas it induces IL-10 production by FLS (37), suggesting that PGE₂ may display anti-inflammatory activity. In this study, PGE₂ dose dependently inhibited COX-2 mRNA and protein expression in FLS, an important enzyme in mediating autoimmune arthritis. The PGE₂ concentration (10⁻⁵ M) at which regulatory effects were clearly evident is much higher than the tissue level found in RA (24,25). However, the action of PGE₂ was also observed at concentrations as low as 10⁻⁸ M, which approximates the physiological concentration of PGE₂ in RA joints. Furthermore, in an inflammatory response, PGE₂ is mainly produced by monocytes and macrophages through the direct cell-to-cell contact. Therefore, it is possible that during the interaction of the macrophages with the synovial fibroblasts or T cells, sufficient amount of PGE₂ to result in a functional modulation of FLS may be secreted *in vivo*, as shown in this paper.

A negative feedback exerted by the COX-2 end products on COX-2 expression itself may provide an important autocrine regulatory mechanism in several cell types (38-41). For example, in porcine aortic smooth-

muscle cells, endogenous and exogenous PGE₂ inhibits COX-2 expression following activation by the fibroblast growth factor-2 (38). PGE₂ also inhibits the LPS- or IL-1β-induced COX-2 expression in J774 macrophages and endothelial cells, respectively (39,40). The inhibition of COX-2 expression by 15-deoxy-PGJ₂, a PGD₂ metabolite, has been reported in FLS (41). In this respect, the results in this study support the negative feedback role of PGE₂ in limiting COX-2 expression and thereby its own synthesis. Overall, overproduced PGE₂ may have the potential to quench the ongoing inflammation within the joints by inhibiting COX-2 production and its end products mediating RA inflammation.

On the other hand, there are contrary reports showing PGE₂ increasing COX-2 expression (42-44). In the airway smooth-muscle cells and microglial cells, PGE₂ up-regulated COX-2 expression (42,43). Moreover, in human FLS, PGE₂ enhanced the COX-2 stability and translation (44). One potential explanation for this discrepancy would be the differential cellular effects of PGE₂ according to the PGE₂ receptor (EP) subtypes. For example, depending on the receptors, the consequence of ligand binding to these receptors can be increased cAMP, decreased cAMP, or a phosphoinositide response (7-10). In addition, most experiments of the latter study (44) were performed in the presence of an endogenous PGE₂ inhibitor, NS-398. In fact, NS-398 could alter the expression of the EP3 and EP4 receptors and thus change the sensitivities to exogenous PGE₂ (45). Therefore, the feedback modulation of COX-2 expression by PGE₂ appears to be influenced by multiple factors including the cell types, the EP receptor subtypes, and the use of an endogenous COX-2 blocker.

In this study, all FLS cell lines obtained from the six different patients strongly expressed the EP2 and EP4 receptors relative to EP1 and EP3. An agonist of the EP2/EP4 receptor, 1-hydroxy-PGE₁, mimicked the PGE₂-mediated COX-2 down-regulation, whereas an EP1/EP3 agonist sulprostone did not. It is documented that PGE₂ rapidly triggers cAMP formation in FLS (37). In this study, experiments using a cAMP analogue and inhibitor showed that the cAMP pathway was the major pathway responsible for the down-regulation of COX-2 by PGE₂. Together, these data suggest that PGE₂ may trigger cAMP accumulation through the activation of EP2/EP4 receptor on FLS, which subsequently suppress IL-1β-stimulated COX-2 production. Again, this scenario is consistent with previous studies showing that PGE₂ blocked the production of pro-inflammatory cytokines and matrix metalloproteases in rheumatoid synoviocytes via cAMP elevating mechanism

(37,46).

In RA synoviocytes, signaling via NF- κ B is involved in regulating the COX-2 expression induced by IL-1 β (47). In this study, both PGE2 and the cAMP inducer caused a dramatic reduction in the IL-1 β -induced NF- κ B-binding activity for COX-2 transcription, while a treatment with DDA completely reversed the IL-1 β -induced NF- κ B-binding activity. These observations suggest that the down-regulation of the COX-2 gene by PGE2 may result from the inhibitory actions of cAMP on NF- κ B activity. Considering that the anti-IL-10 Abs partially reversed the PGE2-mediated COX-2 down-regulation, PGE2 may regulate NF- κ B activity through either indirect or direct means; e.g. the activation of intracellular cAMP signaling via the PGE2/PGE4 receptors, and its associated increase in IL-10, which is a well-known regulator of COX-2 expression and NF- κ B activity (30,48). Still, it is possible that the signal transduction cascades after PGE2 stimulation may adopt different pathways for cAMP formation and IL-10 regulation.

It is quite evident that inducible COX-2 is a key enzyme that increases PGE2 in many inflammatory conditions and the selective COX-2 inhibitor is now an established treatment for RA. Nevertheless, the *in vivo* net effect of PGE2 on RA inflammation should be clarified because the role of PGE2 in the etiopathogenesis of RA has become the subject of recent debate. This study demonstrated that PGE2 dose-dependently reduced the incidence and severity of CIA and anti-CII Abs formation, which supports our data demonstrating the PGE2-mediated suppression of COX-2 and proinflammatory cytokines in the FLS (37). In this respect, overproduced PGE2 may play an anti-inflammatory role *in vivo*, rather than being a simple mediator of RA inflammation.

In conclusion, our data shows a novel mechanism for the action of PGE2 in RA in which PGE2 functions as an autocrine regulator of its own synthesis by inhibiting COX-2 production. PGE2 modulation of COX-2 production was mimicked by a EP2/EP4 receptor agonist, and primarily mediated by a cAMP signal. Anti-inflammatory activity of PGE2 in RA was confirmed in animal models of RA. These results offer a new possibility of manipulating the function of the FLS by modulating the PGE2 receptors and cAMP.

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