

EXTENDED REPORT

EP₂/EP₄ signalling inhibits monocyte chemoattractant protein-1 production induced by interleukin 1 β in synovial fibroblasts

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Background: Besides its proinflammatory properties, prostaglandin E₂ (PGE₂) acts as a regulator of the expression of inducible genes. Inhibition of PGE₂ synthesis might thus result in a paradoxical deleterious effect on inflammation.

Objective: To examine the effect of PGE₂ on monocyte chemoattractant protein-1 (MCP-1) expression in cultured synovial fibroblasts (SF) stimulated with interleukin (IL)1 β .

Methods: MCP-1 expression was assessed in SF stimulated with IL1 β in the presence of PGE₂ or different NSAIDs by RT-PCR or northern blot and immunocytochemistry. Expression of cyclo-oxygenase (COX) isoforms was studied by western blot techniques. The role of PGE₂ receptors (EP) in PGE₂ action was assessed employing EP receptor subtype-specific agonists.

Results: PGE₂ significantly inhibited IL1 β induced MCP-1 expression in SF in a dose dependent manner. IL1 β increased COX-2 and did not alter COX-1 synthesis in SF. 11-Deoxy-PGE₁, an EP₂/EP₄ agonist, reproduced PGE₂ action on MCP-1 expression. Butaprost, a selective EP₂ agonist, was less potent than PGE₂. Sulprostone, an EP₁/EP₃ agonist, had no effect on IL1 β induced MCP-1 expression. Inhibition of endogenous PGE₂ synthesis by NSAIDs further enhanced MCP-1 mRNA expression in IL1 β stimulated SF, an effect prevented by addition of exogenous PGE₂.

Conclusion: Activation of EP₂/EP₄ receptors down regulates the expression of MCP-1 in IL1 β stimulated SF, while PGE₂ pharmacological inhibition cuts off this signalling pathway and results in a superinduction of MCP-1 expression. The data suggest that NSAIDs may intercept a natural regulatory circuit controlling the magnitude of inflammation, which questions their continuous administration in inflammatory joint diseases.

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Prostaglandins are lipid mediators locally increased in the synovial fluid and synovial membrane of patients with inflammatory joint diseases.¹ Prostaglandin E₂ (PGE₂), particularly, contributes to tissue oedema, hyperalgesia, and cytokine production in inflamed tissues.² PGE₂ synthesis takes place in response to cell activation by cytokines, such as interleukin (IL)1 β or tumour necrosis factor α , and its generation accounts for many of the proinflammatory actions induced by these peptides.^{1,3} However, different, and even opposite, effects of PGE₂ have been seen depending on the experimental system tested. Thus, PGE₂ induced effects on vascular smooth muscle tone,⁴ cellular proliferation,⁵ IL6 production,³ or the synthesis of adhesion molecules⁶ are highly variable. These diverse and cell-specific effects of PGE₂ are greatly dependent on the binding of different subtypes of PGE₂ receptors (EP receptors).⁷ Six EP receptor subtypes have been identified in humans, classified into EP₁, EP₂, EP₃, and EP₄ according to their different signalling pathways (reviewed by Narumiya *et al*⁷). Triggering of EP₁ receptors increases calcium mobilisation. Both EP₂ and EP₄ receptors are coupled with cyclic adenosine monophosphate (cAMP) generation. In addition, three different splicing forms of EP₃ receptor mediate the activation of several second messengers, leading to changes in cAMP levels, calcium mobilisation, and activation of phospholipase C.⁷ Different cell responses can be described for the production of inflammatory mediators, depending on the specific receptor subtype expressed at the cell surface.³

Chronic inflammatory diseases are characterised by proliferation of resident cells and infiltration of large numbers of

mononuclear cells into the tissue. Migration of these cells is directed by the release of chemokines at the target tissue.^{8,9} Chemokines are a superfamily of small proinflammatory peptides that, based on structural analysis, can be divided into two subfamilies, C-X-C—mainly chemotactic for neutrophils—and C-C—primarily attracting mononuclear cells.¹⁰ A variety of chemokines are found at high concentrations in joint effusions and cells of pannus, in rheumatoid and in experimental arthritis.¹¹⁻¹⁴ In particular, monocyte chemoattractant peptide-1 (MCP-1) is known to attract monocytes to the joint, where these cells have a decisive contribution to the perpetuation of disease.¹⁵ The number of macrophages in the synovial membrane is one of the better markers for showing local disease activity.^{16,17} In a parallel way, plasma and synovial MCP-1 concentrations closely reflect the severity of joint inflammation in rheumatoid and in antigen induced arthritis.^{11,18,19} In antigen induced arthritis we have found that expression of synovial MCP-1 strongly correlates with leucocyte infiltration into the tissue.²⁰ Strikingly, we also found that pharmacological inhibition of PGE₂ further increases MCP-1 expression and mononuclear cell concentration, in comparison with no treatment.²⁰ These data

Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; COX, cyclo-oxygenase; DCF, diclofenac; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3'-phosphate dehydrogenase; IL, interleukin; MCP-1, monocyte chemoattractant peptide-1; MXC, meloxicam; NSAIDs, non-steroidal anti-inflammatory drugs; PGE₂, prostaglandin E₂; RT-PCR, reverse transcription-polymerase chain reaction; SF, synovial fibroblasts

suggest that PGE₂ may regulate MCP-1 expression, thus playing an important role in controlling mononuclear cell migration to the joint. In this study we have explored this hypothesis using synovial fibroblasts (SF) stimulated with IL1 β , a well recognised proinflammatory cytokine. Our data provide new evidence for an anti-inflammatory role of PGE₂ in SF acting through its EP₂/EP₄ receptors.

METHODS

Culture of SF

SF were obtained as described previously from the knee synovial membranes of healthy New Zealand rabbits or osteoarthritic patients undergoing joint replacement surgery at the Orthopaedic Department of the Fundacion Jimenez Diaz.²¹ Patients were selected for the study only if they had not taken non-steroidal anti-inflammatory drugs (NSAIDs) for at least 30 days before surgery. All studies were performed strictly in accordance with current local regulations. Briefly, human or rabbit synovial explants were exposed to enzymatic digestion with 1.25 mg/ml of trypsin for 1 hour with agitation (Boehringer Mannheim). Collected cells were resuspended in RPMI 1640 medium (Gibco BRL) enriched with 10% fetal calf serum (BioWittaker) and supplemented with 2 μ m glutamine (Gibco BRL), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were cultured at 37°C in 5% CO₂, and used between the sixth and twelfth passages.

Stimulation of SF

SF were seeded onto Petri dishes (Costar) for RNA or total protein extraction experiments. When cells reached confluence, the medium was changed to RPMI without fetal calf serum. After 48 hours, cells were stimulated with vehicle or human recombinant IL1 β (Immugenex Corp) in the presence or absence of PGE₂, 11-deoxy-prostaglandin E₁, butaprost, sulprostone (all from Cayman Chemicals), or the adenylate cyclase (AC) inhibitor MDL-12,330A (Calbiochem) in the indicated concentrations. Where indicated, cells were pre-incubated for 1 hour with diclofenac (DCF; Sigma Chemicals) or meloxicam (MXC; Boehringer Ingelheim) before IL1 β addition.

RNA studies

Rabbit or human SF were harvested and total RNA was obtained by the acid guanidinium thiocyanate-phenol-chloroform method.²² Messenger RNA expression in rabbit SF was analysed by reverse transcription-polymerase chain reaction (RT-PCR)¹¹ and by northern blot²² in human SF. cDNA probes for human MCP-1 (JE*/pGEM-hJE34) and the housekeeping gene glyceraldehyde-3'-phosphate dehydrogenase (GAPDH) were obtained from the American Type Culture Collection. Probes were radiolabelled by nick translation (Boehringer Mannheim) with [³²P]dCTP. For RT-PCR studies, RNA was reverse transcribed and then amplified with a commercial kit (Access RT-PCR System; Promega), employing specific primers for human MCP-1 and GAPDH as described previously.¹¹ Autoradiographs were analysed by scanning densitometry (Biorad).

Immunofluorescence staining

Human SF were grown in eight well Titer-Tek slides (Costar). Quiescent cells were exposed to 10 U/ml IL1 β with or without 10⁻⁶ M PGE₂ for 4 hours. After incubation, cells were washed and fixed in methanol/acetone (50:50 vol/vol) at -20°C. Cells were incubated with 3% bovine serum albumin in phosphate buffered saline (9.1 M dibasic sodium phosphate, 1.7 M monobasic sodium phosphate, 150 M NaCl, pH 7.4) overnight at 4°C. A mouse monoclonal antibody against MCP-1 (10 μ g/ml) (Immugenex Corp) was used as

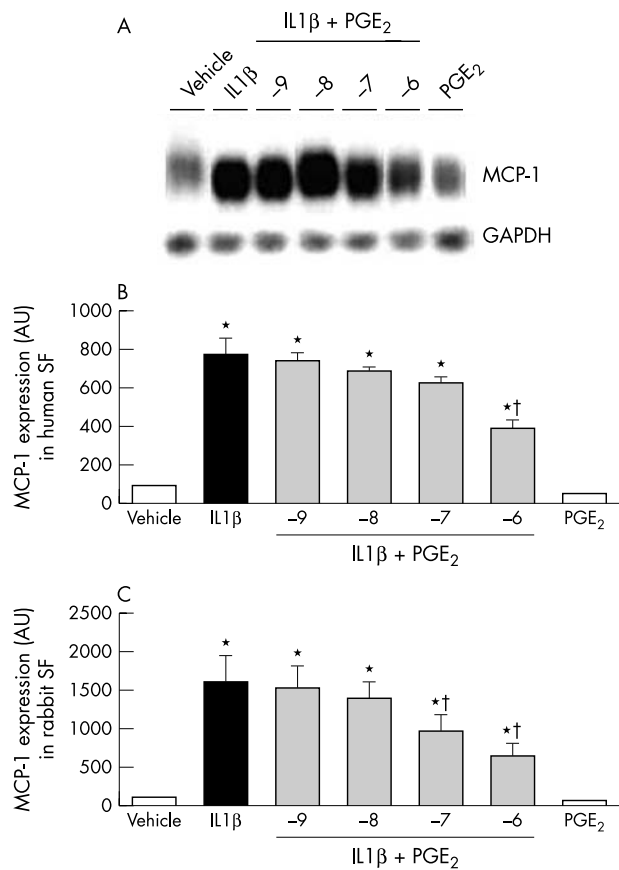


Figure 1 Effect of PGE₂ on MCP-1 expression induced by IL1 β in human and rabbit SF. Cells were incubated for 4 hours with 10 U/ml IL1 β and increasing concentrations of PGE₂. (A) A representative northern blot autoradiograph of four different experiments carried out in human SF. Panels (B) and (C) show the average increase of MCP-1 mRNA levels in human and rabbit SF, respectively, compared with non-stimulated cells (n=4). Data are expressed in arbitrary densitometric units (AU) after correction by mRNA levels of GAPDH, which was employed as housekeeping gene. 10⁻⁶ M PGE₂ did not alter MCP-1 gene expression v vehicle. *p<0.05 v vehicle; †p<0.05 v IL1 β alone.

primary antibody, and a 1:200 dilution of FITC labelled rabbit antimouse IgG was used as a secondary antibody (Sigma). Controls were incubated with non-immune serum or with the secondary antibody alone. Slides were mounted in glycerol and examined under microscope by two "blinded" observers. Images were photographed and printed at equivalent exposures.

PGE₂ assay

The amount of PGE₂ in the conditioned media collected from stimulated rabbit SF was determined by a commercially available enzyme linked immunosorbent assay (ELISA; Assay Designs). Experiments were done in triplicate in two different cell cultures.

Western blot analysis

Human SF were employed for protein determinations owing to the absence of commercially available antibodies against rabbit products appropriate for these studies. For cyclooxygenase-1 (COX-1) and COX-2 determinations, human SF were homogenised with ice cold lysis buffer (1% Nonidet P-40 (Sigma); 0.5% sodium dodecyl sulphate, 0.1 M EDTA, 1 M dithiothreitol, and 1 M phenylmethylsulphonyl fluoride in phosphate buffered saline). The lysates were transferred to Eppendorf tubes and centrifuged at 12 000 g for 10 minutes

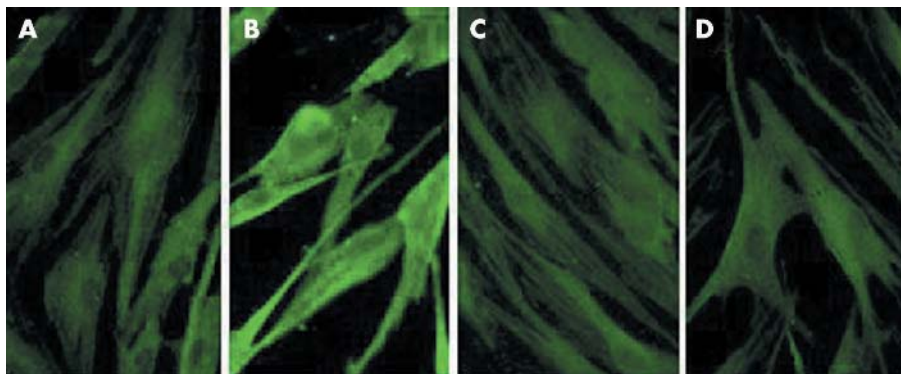


Figure 2 MCP-1 protein detection by immunofluorescence. In untreated human SF, a slight cytoplasmic immunoreactivity to anti-MCP-1 was found (A). Cells treated with 10 U/ml IL1 β for 4 hours showed an intense cytoplasmic fluorescence with the antibody (B). Co-incubation with 10 U/ml IL1 β and 10 $^{-6}$ M PGE $_2$ markedly diminished the MCP-1 signal, in comparison with cells stimulated with IL1 β alone (C). Exposure of SF to 10 $^{-6}$ M PGE $_2$ did not alter the MCP-1 immunostaining pattern in comparison with vehicle (D).

twice. Protein concentrations were determined by the BCA method (Pierce). Equal amounts of protein were run on sodium dodecyl sulphate-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in 0.1 M Tris, pH 7.4, and 0.1 M NaCl containing 0.3% Tween-20 and 6% dry skimmed milk for 60 minutes at room temperature. Then, membranes were incubated overnight with antibodies to COX-1 or COX-2 (both from Santa Cruz Biotech) at 4°C. Detection was done with peroxidase conjugated antibodies, followed by development with a chemiluminescence based method (ECL; Amersham). Controls were performed by immunodetection of α -tubulin in each membrane with a specific antibody (Sigma).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from rabbit SF as previously described.²³ Briefly, cellular nuclei were isolated by precipitation and dissolved in buffer. Total nuclear protein concentration was measured by the BCA method (Pierce). Transcription factor activity was determined by EMSA employing an end labelled [³²P]ATP-NF- κ B consensus oligonucleotide. Nuclear extracts (5 μ g) were equilibrated for 10 minutes in the appropriate binding buffer, and then the labelled probe (0.35 pmol) was added and incubated for 20 minutes at room temperature. To establish the specificity of the reaction, negative controls without cell extracts, and competition assays with a 100-fold excess of unlabelled oligonucleotide were done. DNA-protein complexes were run on a non-denaturing 4% acrylamide gel in Tris-borate. The gel was dried and exposed to an x ray film.

Statistical analysis

Densitometric data are shown as arbitrary units (AU) as a percentage of control values. Results are expressed as the mean (SEM). Comparisons between groups were carried out with the Mann Whitney U test. Values of $p < 0.05$ were considered significant.

RESULTS

Effect of PGE $_2$ on IL1 β induced MCP-1 expression and synthesis

MCP-1 mRNA was studied in rabbit SF by RT-PCR techniques; in human SF northern blot assays were performed, in order to obtain quantitative data. In time curve experiments, the gene expression of MCP-1 was up regulated by 10 U/ml IL1 β in both cell populations, reaching a peak at 4 hours of incubation. For rabbit SF, mRNA levels increased by 910 (230)% at 2 hours*; 1620 (330)% at

4 hours*; 190 (30)% at 9 hours, and by 120 (20)% at 24 hours (n = 4, * $p < 0.05$ v unstimulated cells). Four hours were then established as the optimal period of incubation for the next experiments, in which human or rabbit SF were exposed to 10 U/ml IL1 β in the presence of increasing concentrations of PGE $_2$. Figure 1 shows that similar results for MCP-1 expression were found in human and rabbit cells after incubation with these molecules. Exposure to 10 $^{-6}$ M PGE $_2$ alone did not modify MCP-1 expression in SF. However, addition of PGE $_2$ to 10 U/ml IL1 β -incubated SF resulted in a dose dependent inhibition of MCP-1 expression in comparison with IL1 β stimulated human (fig 1A and B) or rabbit (fig 1C) SF.

To study whether the inhibition of MCP-1 expression was associated with a decrease in the protein production, MCP-1 synthesis was determined by indirect immunofluorescence in human SF using a monoclonal antibody to MCP-1. Starved SF showed a minimal cytoplasmic immunoreactivity to anti-MCP-1 (fig 2A). After 4 hours of stimulation with 10 U/ml IL1 β , enhanced cytoplasmic staining was seen, indicating an increase in MCP-1 synthesis (fig 2B). When SF were co-incubated with 10 U/ml IL1 β and 10 $^{-6}$ M PGE $_2$, a marked diminution in MCP-1 staining was seen as compared with IL1 β stimulated cells (fig 2C). Figure 2D shows that incubation with 10 $^{-6}$ M PGE $_2$ alone did not significantly alter MCP-1 staining in comparison with unstimulated cells. In negative control experiments using unrelated IgG, no staining was detected (data not shown).

Effect of IL1 β on PGE $_2$ production in SF

The negative regulation exerted by PGE $_2$ on IL1 β induction of MCP-1 was particularly remarkable, because PGE $_2$ is quickly synthesised in response to IL1 β , and acknowledged as a key mediator of its actions in different conditions. We therefore studied whether at this period of incubation 10 U/ml IL1 β could increase PGE $_2$ levels in SF culture supernatants and if the synthesis of new COX might participate in this reaction. At 4 hours of incubation, IL1 β caused a 6.5-fold increase in PGE $_2$ release by rabbit SF (fig 3A). When cells were incubated with 10 $^{-6}$ MXC or 10 $^{-6}$ DCF for 1 hour before the addition of IL1 β , PGE $_2$ fell under basal levels, while PGE $_2$ was also almost undetectable in supernatants of cells exposed to either of the NSAIDs alone (fig 3A). In the same way, COX-1 and COX-2 synthesis were studied by western blot analysis in human SF stimulated with IL1 β for 4 hours. At this time, COX-1 protein levels remained unmodified by IL1 β or by the combination of IL1 β plus PGE $_2$, in comparison with vehicle (fig 3B). In contrast, COX-2 concentration significantly rose in IL1 β treated SF (fig 3B). Unlike MCP-1 gene

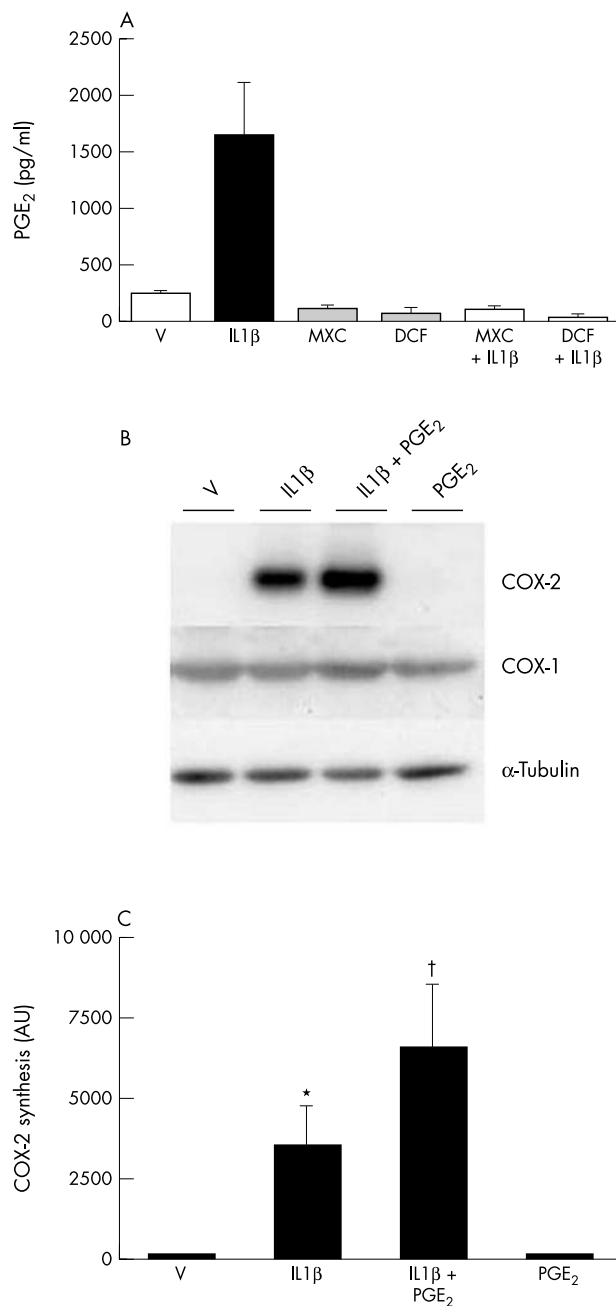


Figure 3 PGE₂ release (A) and COX isoforms (B) in SF incubated with IL1β. (A) PGE₂ release was measured in rabbit SF culture supernatants in triplicate at 4 hours of incubation with 10 U/ml IL1β and/or 10⁻⁶ M MXC or 10⁻⁶ M DCF (pg/ml; n = 2). Untreated cells: 248 (21); 10 U/ml IL1β: 1643 (453); 10⁻⁶ M MXC: 110 (25); 10⁻⁶ M DCF: 39 (0); MXC+IL1β: 104.5 (18); DCF+IL1β: 57.5 (32). (B) Human SF were stimulated with 10 U/ml IL1β and/or 10⁻⁶ M PGE₂ for 4 hours. Upper panel: representative western blots of COX-2, COX-1, and α-tubulin are shown. Lower panel: a densitometric analysis of COX-2 levels expressed in arbitrary units (AU) after correction by α-tubulin (n = 4). *p < 0.05 v vehicle; †p < 0.05 v IL1β alone.

regulation, co-incubation of cells with 10 U/ml IL1β and 10⁻⁶ M PGE₂ resulted in a significantly higher increase of COX-2 synthesis, in comparison with IL1β alone (fig 3B).

Effect of PGE₂ depletion in IL1β induced MCP-1 expression

According to our experiments, although only COX-2 synthesis was stimulated by IL1β, both COX isoforms were

available at the cell cytoplasm at 4 hours of IL1β exposure. To evaluate the effect of endogenous PGE₂ depletion on the IL1β induced MCP-1 expression, we used two non-selective COX-1/COX-2 inhibitors, DCF and MXC, with different COX-2 inhibitor activity.²⁴ Rabbit SF were incubated for 1 hour with MXC or DCF and then stimulated with 10 U/ml IL1β. Figure 4 shows that incubation of cells with 10⁻⁶ M DCF or 10⁻⁶ M MXC alone did not modify MCP-1 gene expression in rabbit SF. However, NSAID pretreated IL1β stimulated rabbit SF showed a more pronounced increase in MCP-1 mRNA expression than cells exposed to IL1β alone. Furthermore, addition of exogenous 10⁻⁶ M PGE₂ to NSAID pretreated, IL1β stimulated SF prevented the up regulation of MCP-1 mRNA levels shown by the combination of NSAIDs and IL1β (fig 4).

Effect of PGE₂ receptor subtype-specific agonists on IL1β induced MCP-1 expression

To establish which of the PGE₂ receptors played a part in the inhibition of MCP-1 expression shown by PGE₂, rabbit SF were incubated with 10 U/ml IL1β together with EP receptor subtype-specific agonists. 11-Deoxy-PGE₁, 10⁻⁶ M, a specific EP₂/EP₄ agonist, suppressed MCP-1 expression evoked by IL1β similarly to PGE₂ (fig 5). Butaprost, 10⁻⁶ M, a specific EP₂ agonist, was less potent than PGE₂ and 11-deoxy-PGE₁ in inhibiting IL1β induced MCP-1 expression. When incubated alone at the same concentrations, 11-deoxy-PGE₁ and butaprost had no effect on MCP-1 expression. In contrast, co-incubation with IL1β and 10⁻⁶ M sulprostone, a selective EP₁/EP₃ agonist, did not have any significant effect on IL1β induced MCP-1 expression (fig 5).

EP₂/EP₄ are G-protein coupled receptors which activate AC and subsequently increase cAMP levels.⁷ An inhibitor of AC, MDL-12,330A, was used to study the role of cAMP as mediator of PGE₂ in the regulation of MCP-1 expression. MDL-12,330A was added to rabbit SF cultures at 10⁻⁵ M, 30 minutes before incubation with 10 U/ml IL1β and 10⁻⁶ M PGE₂. This compound completely abolished the down regulation exerted by PGE₂ over IL1β induced MCP-1 gene expression, confirming that an AC dependent pathway was involved in this PGE₂ action (fig 5). However, incubation of cells with 10⁻⁵ M MDL-12,330A and 10 U/ml IL1β did not result in a superinduction of MCP-1 expression in comparison with IL1β alone.

Role of nuclear factor κB (NF-κB) activation on MCP-1 gene control by PGE₂

The MCP-1 promoter region contains binding sites for several transcription factors including NF-κB.²⁵ Because IL1β is a potent activator of NF-κB translocation, we tested whether the inhibition of MCP-1 expression exerted by PGE₂ was associated with interception of the NF-κB pathway. Figure 6 shows the effect of addition of PGE₂ to IL1β on NF-κB activation in cultured rabbit SF. At 1 hour of incubation, 10 U/ml IL1β provoked a strong activation of NF-κB in comparison with vehicle, as shown by EMSA (lanes 1 and 2). The addition of 10⁻⁶ M PGE₂ to 10 U/ml IL1β stimulated SF significantly reduced the NF-κB binding (lane 3). Furthermore, simultaneous incubation of SF with 10 U/ml IL1β and the EP₂/EP₄ agonist 11-deoxy-PGE₁ at 10⁻⁶ M, reproduced PGE₂ inhibition of IL1β induced NF-κB translocation (lanes 3 and 4). Neither the incubation of SF with PGE₂ nor with 11-deoxy-PGE₁ induced a significant modification of NF-κB activity in comparison with unstimulated SF (lanes 5 and 6).

DISCUSSION

Migration of circulating leucocytes to the joint is a central process in the development and progression of inflammatory

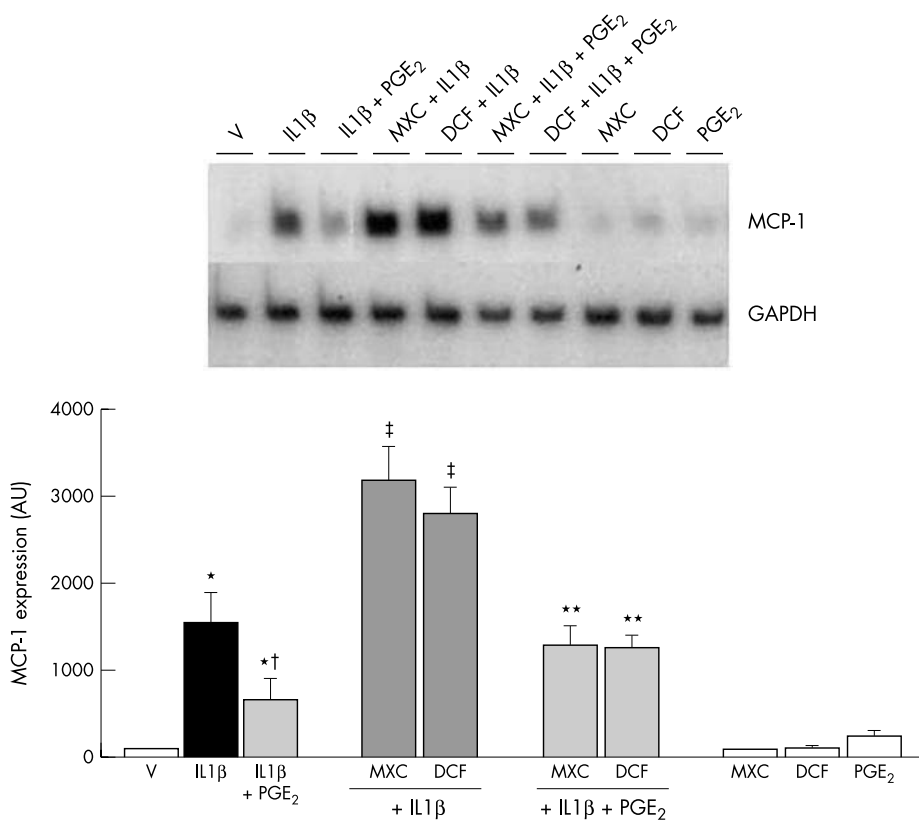


Figure 4 Effect of PGE₂ synthesis inhibition mediated by NSAIDs on MCP-1 expression. Rabbit SF were pre-incubated for 1 hour with 10⁻⁶ M MXC or 10⁻⁶ M DCF, and then 10 U/ml IL1 β and/or 10⁻⁶ M PGE₂ were added for 4 hours. Upper panel shows a representative RT-PCR autoradiograph of MCP-1 and GAPDH. Lower panel shows the analysis of MCP-1 gene expression expressed in densitometric arbitrary units (AU) after normalisation by GAPDH levels (n=4). *p<0.05 v vehicle; †p<0.05 v IL1 β alone; ‡p<0.05 v IL1 β +PGE₂; **p<0.05 v the NSAID+IL1 β .

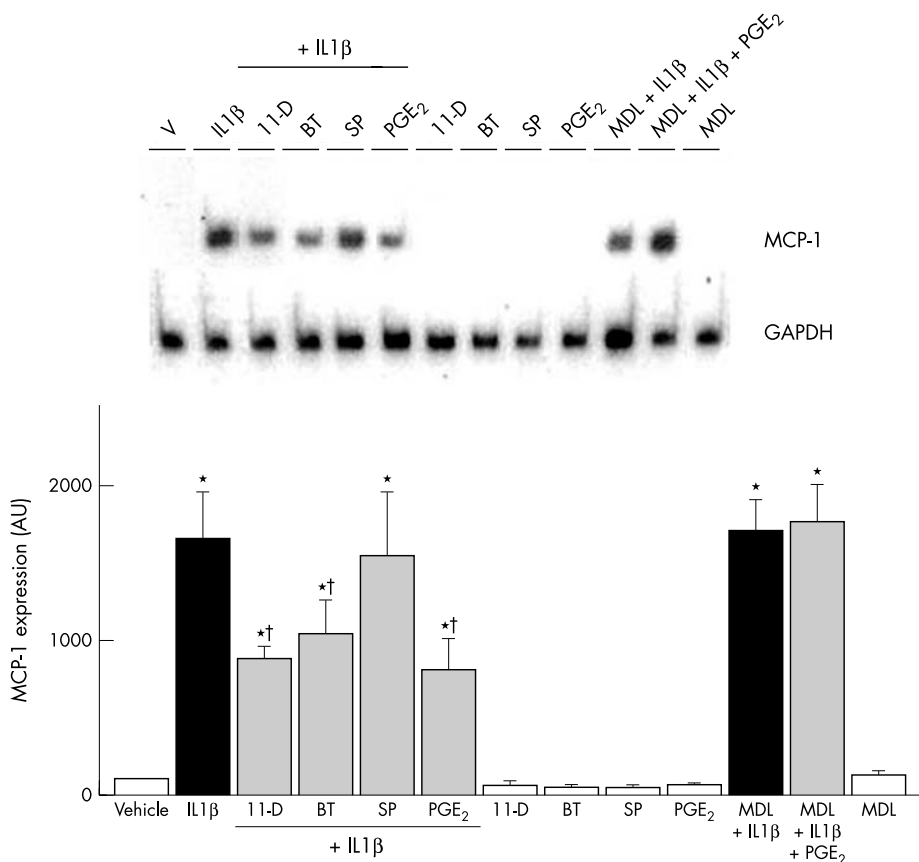


Figure 5 Effect of specific EP receptor-subtype agonists on MCP-1 expression induced by IL1 β . The role of EP receptors in MCP-1 regulation was studied in rabbit SF by RT-PCR, at 4 hours of incubation with 10 U/ml IL1 β and/or the following molecules: the EP₂/EP₄ agonist 11-deoxy-PGE₁ (11-D; 10⁻⁶ M), the EP₂ agonist butaprost (BT; 10⁻⁶ M), the EP₁/EP₃ agonist sulprostone (SP; 10⁻⁶ M), PGE₂ (10⁻⁶ M), and the AC inhibitor MDL-12,330A (MDL, 10⁻⁵ M, 30 minutes preincubation). Upper panel shows a representative autoradiograph of MCP-1 and the housekeeping gene GAPDH. Lower panel shows the analysis for MCP-1 expression in densitometric arbitrary units (AU) relative to that of GAPDH (n=4). *p<0.05 v vehicle; †p<0.05 v IL1 β alone.

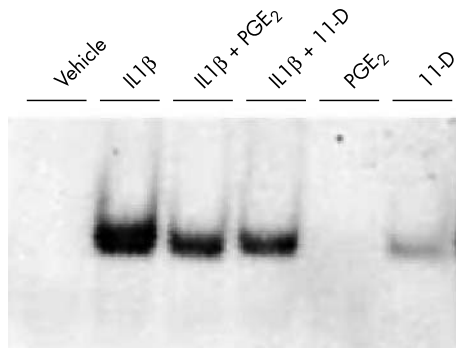


Figure 6 Effect of PGE₂ or the EP₂/EP₄ specific agonist 11-deoxy-PGE₁ (11-D) on NF-κB DNA binding induced by IL1β. Human SF were incubated with 10 U/ml IL1β and/or 10⁻⁶ M PGE₂ or 10⁻⁶ M 11-deoxy-PGE₁ for 60 minutes, and NF-κB activity was measured by EMSA. A representative autoradiograph of four different experiments with similar results is shown.

joint diseases.¹⁵ MCP-1 promotes recruitment of mononuclear cells and their activation in the inflamed synovium.^{13 26 27} It also induces cytokine production by SF, therefore displaying a major role in the interactions between resident and incoming cells which participate in the inflammatory

reaction.²⁸ Proinflammatory mediators locally increased in the rheumatoid synovium, such as IL1, interferon γ , and tumour necrosis factor α , induce MCP-1 expression in both cell populations.^{29 30}

PGE₂ is the eicosanoid achieving higher concentrations in inflamed joints.¹ A complicated network of interactions between PGE₂ and some cytokines has been described. Although the lipid mediator regulates production of these cytokines, the latter can, in turn, stimulate PGE₂ synthesis in most cells.³¹

This work underlines the possibility that despite being considered a key proinflammatory mediator which contributes to joint injury, PGE₂ has an anti-inflammatory role. We have demonstrated that PGE₂ efficiently and dose dependently inhibits IL1 β induced MCP-1 production in SF. Increasing evidence supports the statement that the diverse and specific effects of PGE₂ rely on the cell expression of different EP receptor subtypes. Among these, EP₂, EP₃, and EP₄ have been localised in human SF.³² We have shown that incubation of IL1 β stimulated SF with an EP₂/EP₄ agonist evokes PGE₂ effects on MCP-1 expression. No significant effect was noted for an EP₁/EP₃ agonist, while the effect of a specific EP₂ agonist was less potent than that shown by the EP₂/EP₄ agonist. Activation of EP₂/EP₄ receptors has been associated with cAMP generation.⁷ In our experimental conditions, AC inhibition completely abolished the effect of

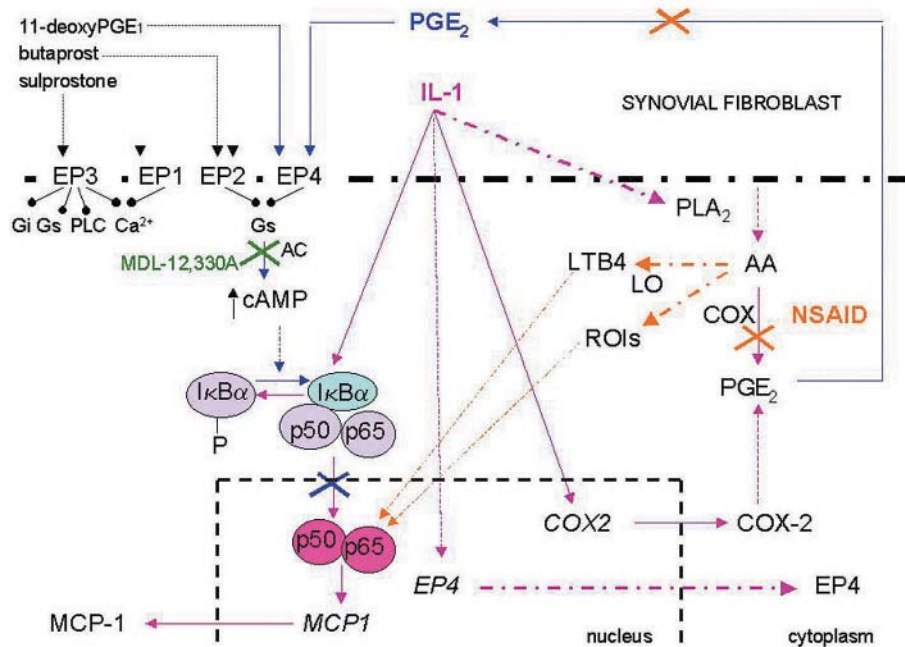


Figure 7 Signalling pathways controlling MCP-1 expression in SF, in which IL1 β and PGE₂ are involved. Mechanisms triggered by IL1 β (pink arrows); PGE₂ dependent signals (blue track); effects of NSAIDs (orange); action of MDL-12,330A, an AC inhibitor (green). Crossed bars: blockade of pathways; continuous lines: mechanisms explored in our study; broken lines: data from published reports. PGE₂ receptors (EP₁ to EP₄) are shown in connection with each specific agonist employed in this study and with their second messengers. IL1 β is one of the strongest activators of SF in inflammation. Many of its actions are mediated by PGE₂ prompt synthesis. This is warranted by (a) activation of phospholipase A₂ (PLA₂) with generation of arachidonic acid (AA), which is the substrate for the action of COX and (b) increased availability of COX-2, whose synthesis is activated by the cytokine. IL1 β promotes recruitment of mononuclear cells to the inflamed joint by the induction of MCP-1 gene expression in SF. IL1 β is an activator of NF-κB, which controls transcription of MCP-1. At the same time, levels of PGE₂ increase in response to IL1 β . When targeting EP₄, PGE₂ could act by tempering inflammation. Addition of PGE₂ or EP₄ agonists leads to a down regulation of MCP-1 expression induced by IL1 β in SF. Thus, IL1 β could initiate lesion producing mechanism as well as restoring mechanisms in the target cell. It may even increase cell sensitivity to PGE₂ by up regulating the expression of EP₄ receptors. EP₄ is coupled with cAMP generation, being the second messenger able to block translocation to the nucleus of NF-κB active components, where they trigger transcription of inducible genes, such as MCP-1. Thus, addition of MDL-12,330A, an inhibitor of AC, to IL1 β plus PGE₂, impairs PGE₂ regulation of MCP-1 gene transcription. Finally, the regulating circuit is intercepted by the employment of NSAIDs owing to the dramatic reduction of PGE₂ levels that these molecules provoke. Furthermore, NSAID blockade of COX may account for deviation of AA metabolism to the production of proinflammatory mediators generated by lipoxygenases (LO), some of which can activate NF-κB.

PGE₂ on MCP-1 expression. Our results are in agreement with recent data supporting the statement that EP₂ and EP₄ transduce anti-inflammatory actions of PGE₂. In brief, it has been demonstrated that activation of these receptors inhibits expression of integrins and synthesis of IL6 in stimulated fibroblasts and in mononuclear cells^{3 6 33}. A recent report suggests that EP₄ activation would also down regulate chemokine expression in tissue infiltrated macrophages.³⁴ In vivo, EP₄ activation has been shown to improve clinical signs of inflammation in an experimental model of colitis.³⁵ Interestingly, we have recently observed that IL1 β induces EP₄ gene expression in SF (data not shown), an effect also found by other authors in cervical fibroblasts.³⁶ The hypothesis might therefore be proposed that PGE₂ dependent responses drawn by EP₄ activation are favoured in the inflamed joint owing to up regulation of the expression of these receptors by IL1 β .

Our data provide new evidence suggesting that a complete counter regulating circuit is started in response to IL1 β generation in the inflammatory environment, directed towards tempering the inflammatory reaction. Figure 7 shows this circuit. Briefly, in our experimental conditions IL1 β was used to reproduce an inflammatory status in SF cultures. The cytokine activated PGE₂ production by SF and also potentially provided a further source of prostanoid synthesis by increasing COX-2 protein levels. In turn, PGE₂, acting through its EP₂/EP₄ receptors, could regulate migration of mononuclear cells to the joint, as suggested by its pronounced down regulation of MCP-1 production by SF. Finally, IL1 β would increase the responsiveness of SF to this PGE₂ anti-inflammatory pathway, through a selective up regulation of EP₄ receptors.

In this work we have also shown that PGE₂ depletion, induced by MXC or DFC, on IL1 β stimulated SF further increased MCP-1 expression in comparison with SF stimulated with IL1 β alone. Although addition of exogenous PGE₂ reversed this NSAID effect, other intercellular pathways should be examined to find out the precise mechanism of action of the drugs, because in this case, inhibition of AC did not result in a similar superinduction of MCP-1 mRNA expression in cells exposed to IL1 β . One interesting possibility might be deviation of arachidonate metabolite production after COX blockade (fig 7); in fact, both accumulation of arachidonic acid and its oxidation by lipoxygenases might result in up regulation of proinflammatory gene products through an NF- κ B dependent pathway.³⁷ Whatever the mechanisms involved, this effect of NSAIDs on SF might explain some inflammatory flares seen during NSAID treatment in arthritic patients.

We must be cautious in the extrapolation of these in vitro results obtained with short term incubations to the clinical setting. Kinetics of both the EP receptor and cAMP mediated signalling pathways are processes tightly regulated. For instance, there seems to be a concentration dependent biphasic curve for cAMP generation yielded by PGE₂, and also a deactivation phenomenon induced by prolonged exposure to the stimulating eicosanoid.³⁸ None the less, our present work has shown similar results to those of our previous in vivo study, which found that NSAID mediated PGE₂ inhibition increased synovial mononuclear cell concentration and MCP-1 expression in arthritic joints,²⁰ resulting in the suggestion that total inhibition of PGE₂ might be non-desirable in chronic inflammation.

One of the possible cellular mechanisms involved in the regulating action of PGE₂ has also been studied in the present work. We have shown that EP₂/EP₄ activation inhibits NF- κ B binding induced by IL1 β . Because MCP-1 is an NF- κ B dependent gene, this action might account for the PGE₂ observed regulation of MCP-1 gene expression (fig 7).

Previous studies have found that cAMP generation inhibits NF- κ B dependent transcription of inflammatory genes in monocytic and endothelial cells.³⁹

In summary, our results show that PGE₂ mediated EP₂/EP₄ activation inhibits MCP-1 expression and synthesis in IL1 β stimulated SF. Blockage of pathways elicited by EP₂/EP₄ by inhibition of PGE₂ synthesis significantly superinduces MCP-1 expression in a proinflammatory setting. Our results suggest that endogenous PGE₂ may control the propagation of inflammation in the inflamed synovium. Thus, chronic administration of NSAIDs might impair the beneficial effects of this autoregulatory circuit.

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