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Microsomal Prostaglandin E Synthase Is Regulated by Proinflammatory Cytokines and Glucocorticoids in Primary Rheumatoid Synovial Cells

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The selective induction of PGE$_2$ synthesis in inflammation suggests that a PGE synthase may be linked to an inducible pathway for PG synthesis. We examined the expression of the recently cloned inducible microsomal PGE synthase (mPGES) in synoviocytes from patients with rheumatoid arthritis, its modulation by cytokines and dexamethasone, and its linkage to the inducible cyclooxygenase-2. Northern blot analysis showed that IL-1b or TNF-$\alpha$ treatment induces mPGES mRNA from very low levels at baseline to maximum levels at 24 h. IL-1b-induced mPGES mRNA was inhibited by dexamethasone in a dose-dependent fashion. Western blot analysis demonstrated that mPGES protein was induced by IL-1b, and maximum expression was sustained for up to 72 h. There was a coordinated up-regulation of cyclooxygenase-2 protein, although peak expression was earlier. Differential Western blot analysis of the microsomal and the cytosolic fractions revealed that the induced expression of mPGES protein was limited to the microsomal fraction. The detected mPGES protein was catalytically functional as indicated by a 3-fold increase of PGES activity in synoviocytes following treatment with IL-1b; this increased synthase activity was limited to the microsomal fraction. In summary, these data demonstrate an induction of mPGES in rheumatoid synoviocytes by proinflammatory cytokines. This novel pathway may be a target for therapeutic intervention for patients with arthritis. The Journal of Immunology, 2001, 167: 469–474.

Inflammation of synovial tissues of patients with arthritis is due, at least in part, to enhanced production of PGs (1). PGs are synthesized by an enzyme cascade initiated by the release of arachidonic acid by a phospholipase. It has become apparent over the last several years that there are distinct pathways by which PGs are formed. Constitutively produced PGs mediating homeostatic functions and PGs produced immediately following cellular activation are synthesized via enzymes expressed under basal conditions, including cytosolic phospholipase A$_2$ (PLA$_2$)$^3$ and cyclooxygenase (COX)-1 (2). In contrast, induction of high level PG production in a time- and tissue-specific manner occurs via a set of synthetic enzymes whose expression is tightly regulated by pathologic and physiologic stimuli. In inflammatory arthritis, there is a marked increase in soluble PLA$_2$ in the joint fluid providing tran-scellular arachidonic acid for eicosanoid biosynthesis (3, 4). Cyto-
solic PLA$_2$ levels are also increased by treatment of cultured synovial cells with IL-1 (5, 6). We and others have shown the dramatic increase in levels of the inducible COX isoform, COX-2, in response to proinflammatory cytokines in synovial tissues and cells of patients with arthritis (6–9). The dominant role for COX-2 in producing proinflammatory PGs associated with arthritis was confirmed in clinical trials demonstrating equal efficacy of specific COX-2 inhibitors and nonsteroidal anti-inflammatory drugs that block activity of both COX isoforms (2).

Among the stable PGs produced in rheumatoid synovia, PGE$_2$ plays an important role. Injection of PGE$_2$ recapitulates the cardinal signs of inflammation via vasodilation with plasma extravasation and sensitization of nociceptors (10). Furthermore, PGE$_2$ stimulates production of matrix metalloproteinases (11) and angiogenesis (12) and inhibits apoptosis of T lymphocytes (13). PGE$_2$ is specifically implicated in the symptoms of arthritis because neutralizing Abs against PGE$_2$ are able to inhibit acute and chronic inflammation in the rat adjuvant arthritis model (14). Previous data point to a selective induction of PGE$_2$ synthesis in inflammation (15–18), suggesting that a PGE synthase (PGES) may be linked to the inducible pathway for PG synthesis.

Until recently, the ability to study regulation of terminal synthase enzymes was hampered by the fact that the PGES enzyme(s) had not been purified. However, two forms of the terminal synthase involved in production of PGE$_2$ were recently cloned and characterized (19–22). Similar to the more proximal PG synthetic enzymes, one isoform is constitutively expressed and unresponsive to proinflammatory stimuli, whereas the other isoform is inducible. The constitutive PGES is expressed in the cytosol (cPGES) under basal conditions in a wide variety of mammalian cell lines and rat tissues (21). Expression is generally unaltered by stimulation with bacterial LPS.

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3 Abbreviations used in this paper: PLA$_2$, phospholipase A$_2$; COX, cyclooxygenase; PGES, PGE synthase; cPGES, cytosolic PGES; mPGES, inducible microsomal PGES; RA, rheumatoid arthritis; AA, arachidonic acid; ASA, acetylsalicylic acid; PGIS, prostacyclin synthase; FLAP, 5-lipoxygenase-activating protein.
The inducible PGES is localized to the microsomal compartment and hence termed mPGES (19, 22). The enzyme was identified as a member of the membrane-associated proteins involved in eicosanoid and glutathione metabolism family of enzymes originally called microsomal glutathione S-transferase 1-like 1 (23). The same sequence was also identified as a gene induced by p53 (24). Jakobsson et al. characterized this enzyme as a human PGES that was dependent on glutathione for its activity and, more important, inducible by IL-1β in human cells (19, 25). Subsequently, Murakami et al. reported that induced PGES activity previously identified in LPS-stimulated rat macrophages actually originated from this enzyme (22). Rat mPGES exhibited a high degree of sequence homology to the human mPGES, and a mouse homologue was also cloned (22). Thorén and Jakobsson described coordinate regulation of mPGES and COX-2 in a human lung cancer cell line (25). Using cotransfection experiments, functional coupling between constitutive cPGES and COX-1 and inducible mPGES and COX-2 has also been demonstrated (21, 22).

The concept of a regulated PG production pathway including both COX-2 and PGES has important implications for understanding inflammation in arthritis. mPGES has not previously been studied in any clinically relevant primary cell type, including synovial cells. We hypothesized that mPGES would be regulated coordinately with COX-2 by proinflammatory cytokines in synoviocytes isolated from patients with rheumatoid arthritis (RA). This pathway could lead to the marked increase in PGE2 observed in inflammatory arthritis.

**Materials and Methods**

**Synoviocyte culture**

Synovial tissue specimens were obtained from patients with RA at the time of total joint replacement surgery. Tissues were minced and digested for 4 h with 4 mg/ml collagenase (type I; Worthington Biochemical, Freehold, NJ) in DMEM at 37°C in 5% CO2. The dissociated cells were plated in 75-cm² flasks containing DMEM supplemented with 10% human AB serum (BioWhittaker, Walkersville, MD), 10% FBS (Life Technologies, Rockville, MD), penicillin (100 U/ml), and streptomycin (100 μg/ml). When they reached 95% confluency, they were harvested with trypsin/EDTA (Life Technologies) and adjusted to 2 × 10⁶ cells/ml.

**Determination of eicosanoid profile**

Cells were labeled overnight with 3H-labeled arachidonic acid (AA) (1 μCi, sp. act. 76–100 μCi/mmol; DuPont/New England Nuclear, Boston, MA). Unincorporated label was removed by washing. Some cells were examined under unstimulated conditions. Some cells were treated with IL-1β (10 ng/ml) with or without aspirin (1 mM) or the selective COX-2 inhibitor NS-398 (2.5 mM) added simultaneously. Medium was collected for 10 min before harvesting for eicosanoid analysis.

**RNA preparation and analysis**

Total RNA was prepared from synoviocytes using TRI-Reagent (Molecular Research Center, Cincinnati, OH). For Northern blot analysis, 20 μg of total RNA was subjected to electrophoresis through a 1.5% agarose-4% formaldehyde gel and transferred onto a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH). To generate a probe for mPGES, the human mPGES coding sequence of 792 bp (19) was amplified from the pTT73 Pac vector, purified, and 32P-labeled by the random prime method (Random Primed DNA Labeling Kit; Boehringer Mannheim, Mannheim, Germany). A probe for G3PDH was synthesized by random prime method from a cDNA fragment of human G3PDH generated by PCR using primers as described below. Membranes were hybridized in 50% formamide at 42°C for 24 h with 9 × 10⁶ cpm/mg cDNA probe. Washing was performed twice with 2× SSC, 0.1% SDS at room temperature and twice for 15 min in 0.1× SSC, 0.1% SDS at 65°C. The membranes were exposed to Kodak XAR film for 48 h at −80°C (Kodak, Rochester, NY).

The cDNA was prepared by reverse transcription of 5 μg total RNA in 50 μl containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1 mM MgCl2, 1 mM DTT, 0.4 mM dNTPs, 2 μM random hexamer primers (Life Technologies), 0.1 μg/ml RNAse inhibitor (Life Technologies), and 8 μl Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reaction mixtures were incubated at 22°C for 10 min, at 42°C for 30 min, and at 95°C for 5 min. Complementary DNA was diluted 10-fold and used for PCR.

The PCR was performed in 50 μl containing 5 μl cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 100 μM dNTPs, 0.05 μg/ml Taq polymerase (Life Technologies), 40 μM (for mPGES and prostacyclin synthase (PGIS), 2 mM for COX-2 and G3PDH, and 2.5 mM for cPGES). The oligonucleotide primers used were as follows: for mPGES, sense 5′-ATGCCTGGACACAGCTGT-3′ and antisense, 5′-TCAAGTTGGCGCAGCCG-3′; for cPGES, sense, 5′-ATCGCAGCTCGTTCTGCA-3′ and antisense, 5′-TAATTCGAGATCTGCCC-3′; for COX-2, sense, 5′-CTCAAATGAGATTGTGGGAAAATTGCT-3′ and antisense, 5′-AGATCATCTCTGGTGGATTCT-3′; for PGIS, sense, 5′-GGAGCAATAGCTGGAGAGTTAC-3′ and antisense, 5′-ATCCGTAGGGTTCGAAATCG-3′; and for G3PDH, sense, 5′-CCACCATGTCAGTTTGAGCACA-3′ and antisense, 5′-TCTGACGGCAGCTGTTGCC-3′. Cycling conditions were as follows: denaturation at 94°C for 2 min; annealing at 70°C for mPGES and PGIS, 65°C for COX-2, 60°C (G3PDH), or 50°C (cPGES) for 1 min; extension at 72°C for 1 min; 25 cycles for G3PDH and 30 cycles for COX-2, cPGES, and PGIS. All PCR conditions were adjusted to assure that product formation was on the linear portion of a cycle curve.

**Immunoblot analysis**

Synoviocytes were trypsinized, pelleted, washed in ice-cold phosphate-buffered saline, and resuspended in lysis buffer (10 mM HEPES (pH 7.9) at 4°C, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors). Protein concentration of the lysates was determined by the Bradford method (Bio-Rad). The lysates were normalized to protein concentration.

**PCR activity assay**

To determine PGES activity in whole cells, primary RA synoviocytes were cultured in six-well plates in complete medium. After they reached confluence, cells were washed with 2 ml PBS, and the medium was changed to DMEM supplemented with 2% FBS and 1 mM ASA to inhibit enzymatic activity of endogenous COXs. Cells were treated with IL-1β for 24 or 72 h, washed with 2 ml PBS, and medium was exchanged. In some experiments, cells were pelleted and frozen in liquid N2 for fractionation.

For analysis of PGES activity in intact synoviocytes, plates were placed on ice and 7.05 μg PGH2 (Cayman Chemicals) was added. The reaction was initiated by adding 10 μl polymerization buffer containing NADPH, NADP, ADP, ATP, 2 μg/ml bovine serum albumin, 0.25 μM [14C]PGH2, 5 μg/ml COX-2, and 1 μg/ml NADPH oxidase. At various time points, the reaction was terminated by adding 40 μl of 10% trichloroacetic acid. Supernatant was assayed for [14C]PGE2 by liquid scintillation counting. Most of the [14C]PGE2 was recovered in the supernatant. PGES activity was determined by the Bradford method (Bio-Rad).
was stopped by adding 100 μl of 400 mM FeCl₂-4 mM citric acid at the times indicated in the figure legends. Incubation of cells with acetone for 5 min served as negative control. Measurement of PGE₂ was performed by enzyme-linked immunoassay (Cayman Chemicals).

Evaluation of PGES activity in microsomal fractions was performed as previously described (25). In these experiments, PGE₂ was measured by reversed-phase HPLC with UV detection.

Results

**Preference induction of PGE₂ by IL-1β in primary synoviocytes**

Under basal conditions, RA synoviocytes produced low levels of PGs from endogenous AA released by treatment with calcium ionophore, mostly PGI₂ (Fig. 1). To determine changes in the profile of stable PGs after induction of COX-2, we treated synoviocytes with aspirin (1 mM) to inhibit constitutive COX-1. Aspirin was removed, and cells were treated for 6 h with IL-1β, which we have previously shown to be sufficient to induce COX-2 protein (9). Levels of PGI₂ were increased, but there was a proportionally greater induction of PGE₂ (Fig. 1).

To confirm that substrate was from a COX-2-dependent pathway, we incubated synoviocytes with either aspirin (1 mM), which inhibits both COX-1 and COX-2, or NS-398 (3 μM), a specific COX-2 inhibitor, during a 16-h incubation with IL-1β or TNF-α. These experiments were performed three times using cells from three different RA patients. Complete inhibition of PGE₂ in conditioned medium was seen in the aspirin- and NS-398-treated cells, demonstrating that substrate for PGE₂ production was generated by a COX-2-dependent pathway.

**mPGES mRNA expression is induced by pro-inflammatory cytokines**

A specific increase in PGE₂ could be explained by induction of a specific PGE synthase. To evaluate that possibility, we examined expression of the inducible mPGES. Treatment with IL-1β led to a marked increase of mPGES mRNA from very low levels at baseline to maximum levels at 24 h, as shown by Northern blot analysis (Fig. 2A). Levels were increased by 4 h after treatment with IL-1β, and high level mRNA expression was sustained for up to 72 h after treatment. TNF-α treatment for 24 h also resulted in an enhanced mPGES mRNA signal, but to a lesser degree than with IL-1β (Fig. 2B).

**mPGES, but not cPGES, mRNA is regulated similarly to COX-2 by IL-1β and dexamethasone**

As previously documented, COX-2 mRNA levels were up-regulated by IL-1β (Fig. 3). IL-1β-induced COX-2 and mPGES trans
Nonenzymatic degradation of PGH2 to PGE2 is minimal in our results were obtained using synoviocytes from three patients with RA. Maximum expression of mPGES was at 24 h and sustained to 72 h. Similar results were obtained using synoviocytes from three patients with RA.

The increase of mPGES protein was associated with a 3-fold increase of PGES activity in microsomal fractions of IL-1β-treated synoviocytes (Fig. 5). In accordance with localization of mPGES protein to the microsomal fraction (Fig. 5), the microsomal fraction (Fig. 5).

Discussion

This manuscript is the first to describe expression and regulation of mPGES in primary rheumatoid synovial cells. This system is relevant for understanding pathogenic mechanisms and potential therapeutic targets in RA. There is abundant data to implicate PGE2 as a mediator of inflammation in arthritis (1). In addition, PGE2 affects tissue remodeling in the setting of chronic inflammation and has important effects on cells that mediate immune and inflammatory processes. Our data provide further support for the hypothesis that expression of the inducible enzymes of the PGE2 production pathway in vivo is mediated by proinflammatory cytokines in the milieu of the arthritic joint.

Similar to the COX enzymes, it appears that the PGE2 enzymes function as a pair of isoforms (21, 22). The experiments presented here demonstrate that the mPGES is the inducible isoform responsible for specific up-regulation of PGE2 in response to inflammatory stimuli in synovial tissues. In addition to increased mRNA and protein expression, we show that endogenous PGES activity is increased in intact cells after treatment with IL-1β. Although both cPGES and mPGES mRNA are present in synoviocytes, only mPGES expression is regulated. Induction of PGES activity by IL-1β suggests the mPGES is responsible. The observation that PGES activity is increased in the microsomal fraction further implicates mPGES rather than cPGES as mediating increased PGE2 production. Moreover, we demonstrated that mPGES expression occurs in a pattern generally similar to COX-2 with expression increased by proinflammatory cytokines and blocked by corticosteroids. These observations suggest a functional linkage between COX-2 and mPGES. This idea is supported by the finding that specific COX-2 inhibitors reduce production of PGE2 more than other stable PGs (27). Finally, our results provide an additional target by which glucocorticoids exert anti-inflammatory effects, because dexamethasone completely suppressed the induction of mPGES by IL-1β.

Although COX-2 and mPGES expression vary similarly in response to the same stimuli, there are differences in the specific

![FIGURE 5](image_url) The inducible PGES is localized to the microsomal fraction. Cytosolic and microsomal fractions of synoviocytes from three different patients treated for 24 or 72 h with IL-1β (1 ng/ml) were isolated. Immunoblotting was performed using anti-PGES antiserum. Positive control lanes were bacterially expressed PGES containing 1 μg total protein, whereas other lanes contained 5 μg protein. C, Cytosolic fraction; M, microsomal fraction.

![FIGURE 4](image_url) mPGES protein is increased after treatment with IL-1β. Synoviocytes were treated with IL-1β (1 ng/ml) for varying times. mPGES and COX-2 protein expression increased after treatment with IL-1β. Peak COX-2 expression occurred by 4–8 h and begins to decline by 24 h; maximum expression of mPGES was at 24 h and sustained to 72 h. Similar results were obtained using synoviocytes from three patients with RA.

![FIGURE 6](image_url) Stimulation of synoviocytes with IL-1β increases PGES activity. PGES activity assay was performed by adding exogenous PGH2 (10 μM) to wells without cells (to assay for nonenzymatic degradation of substrate), untreated synoviocytes, or synoviocytes treated with IL-1β for 24 h. Reactions proceeded on ice over 5 min. PGE2 was assayed by enzyme-linked immunosassay. Only cells treated with IL-1β demonstrated significant PGES activity. The data represent three experiments using cells from different patients with RA. *p < 0.01 comparing 0 and 5 min by Students t test. C denotes samples incubated for 5 min in acetone without addition of PGH2.
tathione. PGE2 product was detected by reversed-phase HPLC. COX-2 inhibitors have decreased gastrointestinal toxicity compared to traditional nonsteroidal anti-inflammatory drugs, it is clear that there are adverse effects due to inhibition of COX-2 (2). For example, specific inhibition of COX-2 inhibits production of renal and systemic PGI2 (32, 33). Consistent with an important physiologic role for COX-2 in the kidney, specific COX-2 inhibition can lead to altered excretion of sodium, edema, and elevated blood pressure (34). In addition, specific inhibition of COX-2 alters the balance between platelet-derived thromboxane A2 and endothelial PGI2, leading to altered vascular tone and perhaps to an increased risk of thrombosis (35, 36). Thus, a more selective modulation of the prostanooid pathway is desirable. It is currently unclear how much of the inflammation in human arthritis is mediated by PGE2; indeed, mice with a targeted deletion of the prostacyclin receptor have reduced pain and inflammation (37). Nevertheless, these data suggest a novel pathway that may be a target for therapeutic intervention for patients with arthritis.

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FIGURE 7. IL-1β-stimulated PGES activity is localized to the microsomal fraction. Microsomal fractions were isolated from untreated RA synoviocytes or synoviocytes treated for 24 or 72 h with IL-1β (1 ng/ml). Samples with increasing protein concentrations were incubated for 1 min on ice with PGG2 (11.3 μM) in a buffer containing 2.5 mM reduced glutathione. PGE2 product was detected by reversed-phase HPLC.

Timing for induction. In keeping with the differences in the time course of COX-2 and mPGES expression after stimulation with IL-1β, the promoter of the mPGES gene lacks many of the elements usually associated with cytokine-inducible genes (20). Examination of a 651-bp promoter region revealed the presence of GC boxes, barbie boxes, and an aryl hydrocarbon regulatory element but the mPGES promoter lacked a TATAAA box and other known transcriptional elements. In addition to transcriptional stimulation by IL-1β, promoter reporter constructs demonstrated enhanced transcription in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin, known to stimulate transcription via aryl hydrocarbon regulatory elements. Transcription was inhibited by phenobarbital, presumably acting via the barbie boxes (20). COX-2 expression is regulated by posttranscriptional mechanisms (28, 29). The 3′ region of mPGES lacks the AUUUAA instability sequences found in the COX-2 gene, suggesting another possible difference in regulatory mechanisms of the two enzymes (20).

Similar to our findings, cytokine induction of mPGES in human A549 cells and rat macrophages was suppressed by dexamethasone (22, 25). Moreover, a functional linkage between mPGES and COX-2 was proven in transfected human embryonic kidney 293 cells, which stably expressed both enzymes. In those experiments, functional linkage could not be explained solely on the basis of subcellular localization because mPGES, COX-1, and COX-2 were all expressed in a perinuclear distribution. The basis for functional linkage remains unclear at the current time. However, mPGES is a member of a superfamily of enzymes involved in eicosanoid metabolism which includes 5-lipoxygenase-activating protein (FLAP). FLAP is involved in the transfer of AA to 5-lipoxygenase to achieve efficient leukotriene production (30). It is conceivable that mPGES could facilitate interaction with COX-2 by similar mechanisms. It has been noted that transfection of FLAP into certain cell lines increases PGE2 synthesis and COX-2 expression (31).

Our findings have considerable clinical relevance for understanding and treating inflammatory arthritis. Although specific COX-2 inhibitors have decreased gastrointestinal toxicity compared to traditional nonsteroidal anti-inflammatory drugs, it is

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