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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 PGE2 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-1β or TNF-α treatment induces mPGES mRNA from very low levels at baseline to maximum levels at 24 h. IL-1β-induced mPGES mRNA was inhibited by dexamethasone in a dose-dependent fashion. Western blot analysis demonstrated that mPGES protein was induced by IL-1β, 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-1β; 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.

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 A2 (PLA2)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 PLA2 in the joint fluid providing transcellular arachidonic acid for eicosanoid biosynthesis (3, 4). Cytosolic PLA2 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, PGE2 plays an important role. Injection of PGE2 recapitulates the cardinal signs of inflammation via vasodilation with plasma extravasation and sensitization of nociceptors (10). Furthermore, PGE2 stimulates production of matrix metalloproteinases (11) and angiogenesis (12) and inhibits apoptosis of T lymphocytes (13). PGE2 is specifically implicated in the symptoms of arthritis because neutralizing Abs against PGE2 are able to inhibit acute and chronic inflammation in the rat adjuvant arthritis model (14). Previous data point to a selective induction of PGE2 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 PGE2 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.
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 PGE_2 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-ml flasks containing DMEM supplemented with 10% human AB serum (BioWhitaker, 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 passed 1:3 into 175-ml flasks.

Cells were used between the third and seventh passage. After medium change to DMEM supplemented with 2% FBS, 1% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml), cultures were treated as indicated in the figure legends using human recombinant IL-1β and TNF-α (Genzyme, Cambridge, MA, or R&D Systems, Minneapolis, MN) or dexamethasone (Sigma, St. Louis, MO).

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 unstressed conditions. Some cells were cultured with acetylsalicylic acid (ASA; aspirin) (1 mM) for 30 min to irreversibly inhibit constitutive COX-1, washed extensively to remove the ASA, then treated with IL-1β (1 ng/ml) for 6 h to induce COX-2. All cells were then treated with the calcium ionophore A23187 (2.5 µM) 30 min before harvesting to stimulate activity of PLA_2.

Radiolabeled AA and eicosanoid metabolites were extracted from the medium using a Sep-Pak cartridge (waters, Milford, MA). Lipid extracts were subjected to reversed-phase HPLC as previously described (26). Radiometric analysis was determined online using a Radiomatic Flo-One Beta Detector (Packard, Downers Grove, IL). PG products were identified by comparison with the elution of known standards.

To determine whether PG_E_2 production was dependent on COX-2, synoviocytes were treated in the presence or absence of IL-1β (1 ng/ml) or TNF-α (10 ng/ml) with or without aspirin (1 mM) or the selective COX-2 inhibitor NS-398 (3 µM) added simultaneously. Medium was collected after 16 h and analyzed for PGE_2 by enzyme-linked immunosay (Cayman Chemicals, Ann Arbor, MI). Cells were lysed, and total protein was determined by the Bradford method (Bio-Rad, Hercules, CA). PGE_2 levels were normalized to protein concentration.

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 pT7-T7 Pac vector, purified, and 32P-labeled by the random primer 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 x 10^6 cpm/ml cDNA probe. Washing was performed twice for 10 min in 2 x SSC, 0.1% SDS at room temperature and twice for 15 min in 0.1 x 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, 6 mM MgCl_2, 1 mM DTT, 0.4 mM dNTPs, 2 µM random hexamer primers (Life Technologies), 0.1 µg RNase inhibitor (Life Technologies), and 8 µM 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/µl Taq polymerase (Life Technologies), 0.04 µg/ml primers (Life Technologies), and 10% DMSO in appropriate concentrations (1 mM 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'-AGTGCCTGGCAGCATGCT-3' and antisense, 5'-TCAGGATTCTGGCCCG-3'; for cPGES, sense 5'-ATGACCTGGCTCTGGCCCG-3' and antisense, 5'-TACCCAGCATGAGTCC-3'; for COX-2, sense 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' and antisense, 5'-AGATCATCTCCTGCGGTATCTT-3'; for PGIS, sense 5'-GGAGAAATGAGTTGGAGATGTTAC-3' and antisense, 5'-ATCCGTACGGTTCCAGAATTG-3'; and for G3PDH, sense 5'-CCACCTGATGGCCCATAATTTG-3' and antisense, 5'-TCTAGACGCGGCGTGTGTTCC-3'. Cycling conditions were as follows: denaturing at 94°C for 2 min; annealing at 70°C (mPGES and PGIS), 65°C (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 mPGES, COX-2, cPGES, and PGIS. All PCR conditions were adjusted to ensure 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], 0.1 mM EDTA, 1 mM MgCl_2, 1 mM DTT, 0.4 mM dNTPs, 2 µM random hexamer primers (Life Technologies), and MgCl_2 in appropriate concentrations (1 mM 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'-AGTGCCTGGCAGCATGCT-3' and antisense, 5'-TCAGGATTCTGGCCCG-3'; for cPGES, sense 5'-ATGACCTGGCTCTGGCCCG-3' and antisense, 5'-TACCCAGCATGAGTCC-3'; for COX-2, sense 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' and antisense, 5'-AGATCATCTCCTGCGGTATCTT-3'; for PGIS, sense 5'-GGAGAAATGAGTTGGAGATGTTAC-3' and antisense, 5'-ATCCGTACGGTTCCAGAATTG-3'; and for G3PDH, sense 5'-CCACCTGATGGCCCATAATTTG-3' and antisense, 5'-TCTAGACGCGGCGTGTGTTCC-3'. Cycling conditions were as follows: denaturing at 94°C for 2 min; annealing at 70°C (mPGES and PGIS), 65°C (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 mPGES, COX-2, cPGES, and PGIS. All PCR conditions were adjusted to ensure that product formation was on the linear portion of a cycle curve.

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was stopped by adding 100 μl of 400 mM FeCl$_2$-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 PGE$_2$ was performed by enzyme-linked immunoassay (Cayman Chemicals).

Evaluation of PGES activity in microsomal fractions was performed as previously described (25). In these experiments, PGE$_2$ was measured by reversed-phase HPLC with UV detection.

Results

**Preferential induction of PGE$_2$ 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 PGI$_2$ (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 PGI$_2$ were increased, but there was a proportionally greater induction of PGE$_2$ (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 PGE$_2$ in conditioned medium was seen in the aspirin- and NS-398-treated cells, demonstrating that substrate for PGE$_2$ production was generated by a COX-2-dependent pathway.

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

A specific increase in PGE$_2$ 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 tran...
expression and regulation of mPGES in RA synoviocytes

Nonenzymatic degradation of PGH2 to PGE2 is minimal in our results were obtained using synoviocytes from three patients with RA. 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.

Cytokine effects on mPGES and COX-2 protein expression

mPGES and COX-2 protein expression followed the same pattern as shown above for mRNA (Fig. 4). There was an expected sharp increase in COX-2 protein after IL-1β treatment. Peak expression of COX-2 was more rapid than for mPGES, and levels were beginning to decline by 24 h after treatment (Fig. 4).

To determine the localization of mPGES, differential Western blot analysis for the microsomal and the cytosolic fractions was performed. Induced expression of mPGES protein was limited to the microsomal fraction (Fig. 5).

IL-1β induces mPGES activity

The increase of mPGES protein was associated with a 3-fold increase of PGES activity in intact primary synoviocytes (Fig. 6), thus indicating that the detected mPGES protein is a catalytically functional PGES enzyme. Synovial cells were cultured in the presence of aspirin to achieve irreversible inhibition of both constitutive COX-1 and any COX-2 induced by IL-1β. Aspirin acetylation of COX prevents the entry of AA to the catalytic site of the enzyme. Because endogenous PGH2 synthesis is blocked, we measure PGES activity by conversion of exogenous PGH2 to PGE2. Nonenzymatic degradation of PGH2 to PGE2 is minimal in our experimental conditions, as shown in the control wells without cells. In accordance with localization of mPGES protein to the microsomes, we observed a time-dependent increase in PGES activity in microsomal fractions of IL-1β-treated synoviocytes (Fig. 7).

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 PGES enzymes function as a pair of isozymes (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
tathione. 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 TATAA box and other GC boxes, barbie boxes, and an aryl hydrocarbon regulatory element. Transcription was inhibited by phenobarbital, 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 AUUUA 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 transplanted 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 with 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 PGII2 (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 PGII2, 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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References


