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INVOLVEMENT OF NUCLEAR FACTOR κB IN THE REGULATION OF CYCLOOXYGENASE-2 EXPRESSION BY INTERLEUKIN-1 IN RHEUMATOID SYNOVIOCYTES

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Objective. **To evaluate involvement of the transcription factor nuclear factor κB (NF-κB) in the increased expression of cyclooxygenase-2 (COX-2) stimulated by interleukin-1β (IL-1β) in primary rheumatoid synoviocytes.**

Methods. **We treated early-passage rheumatoid synoviocytes with IL-1β and examined the time course of NF-κB translocation to the nucleus by Western blot analysis, as well as NF-κB binding to the COX-2 promoter/enhancer by electrophoretic mobility shift assay. We correlated the time course of NF-κB binding with expression of COX-2 messenger RNA (mRNA) and protein. Synoviocytes were then treated with either sense or antisense phosphorothioate-modified oligonucleotides derived from the transcription start site of the human NF-κB p65 RNA. We analyzed NF-κB binding to the COX-2 promoter and COX-2 protein levels after these treatments.**

Results. **IL-1β rapidly stimulated the translocation of the p65, p50, and c-*rel* NF-κB subunits from the cytoplasm to the nucleus. Electrophoretic mobility shift assay demonstrated binding to 2 NF-κB sites within the COX-2 promoter/enhancer, with a time course identical to that of nuclear localization of NF-κB. Supershift analysis revealed that binding activity was due primarily to the p65–p50 heterodimer and the p50 homodimer. With appropriate lag time after NF-κB binding, COX-2 mRNA and protein were increased. Pretreatment of RA synoviocytes with NF-κB p65 antisense oligonucleotides resulted in decreased binding to the COX-2 promoter and decreased COX-2 protein expression.**

Conclusion. **These data demonstrate that signaling via the NF-κB pathway is involved in regulation of COX-2 expression induced by IL-1β in RA synoviocytes.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by proliferative and invasive synovitis. Rheumatoid synovial tissues release large quantities of prostaglandins (PGs), mainly PGE_2, $PGF_{2\alpha}$, prostacyclin, and thromboxane, that are important mediators of inflammation (1–4). PGE_2 may also play a key role as a mediator of erosion of cartilage and juxtaarticular bone, and induction of angiogenesis (5–7).

PG synthesis is initiated by release of arachidonic acid from cell membranes (8,9). Arachidonate is converted to PGH_2 by cyclooxygenase (COX; prostaglandin H synthase), the central enzyme in the PG synthetic pathway (10). COX-1 is the constitutively expressed isoform of COX. In contrast, COX-2 is expressed only at low levels under basal conditions, but is markedly induced by proinflammatory or mitogenic stimuli and suppressed by glucocorticoids (11–17). High levels of immunoreactive COX are present in RA synovia as compared with synovia from patients with osteoarthritis or from normal subjects (18). The COX-2 isoform is detectable by specific immunohistochemical analysis in vivo in human RA synovial tissues (16). Primary explanted rheumatoid synovial tissues express both COX-1 and COX-2. After treatment with the proinflammatory cytokine interleukin-1β (IL-1β), expression of COX-2, but not COX-1, messenger RNA (mRNA) and peptide is markedly increased. Dexamethasone eliminates COX-2, but not COX-1, expression in unstimulated explants. Dexamethasone also dramatically inhibits IL-1–induced increases in COX-2 expression. COX-2 regulation by IL-1β and dexamethasone in cultured

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rheumatoid synovial fibroblast-like cells (synoviocytes) parallels the findings in primary explants (16).

The proinflammatory cytokine IL-1 is present in high levels in synovial fluid and peripheral blood of patients with RA (19), and systemic levels of IL-1β correlate with disease activity (20). The majority of IL-1β present in inflamed rheumatoid synovia is produced by macrophages; however, synoviocytes also synthesize IL-1 (21). Finally, injection of IL-1 into rabbit knee joints produces inflammatory synovitis (22,23). IL-1 exerts its effects by binding to the type I IL-1 receptor (IL-1RI) (24,25). The IL-1RI signaling pathway is incompletely understood (26,27). Nevertheless, it is clear that IL-1–induced activation of the transcription factor nuclear factor κB (NF-κB) is a major signaling pathway for IL-1–induced gene expression (27).

NF-κB activity is mediated by a family of transcription factor subunits that bind DNA as hetero- or homodimers. The subunits are present in the cytoplasm in inactive form, bound to inhibitory proteins such as MAD3/IκBα. A variety of extracellular stimuli, including IL-1, induce degradation of IκB. Dissociation of NF-κB from cytoplasmic inhibitors is followed by translocation of the complex to the nucleus, then binding to specific DNA sequences (28). NF-κB–responsive elements are required for the transcriptional regulation of a number of mediators of immune and inflammatory processes (29).

The promoter region of the human COX-2 gene contains 2 motifs with sequence similarity to the consensus binding site for NF-κB (30). Since the IL-1β/NF-κB pathway is of significance in the coordinate regulation of inflammatory processes and this transcriptional pathway has not been examined for COX-2, we investigated the role of NF-κB in the regulation of the human COX-2 gene in RA synoviocytes.

MATERIALS AND METHODS

Synoviocyte culture. Synovial tissues were obtained from patients with RA, at the time of total joint replacement surgery. Tissues were minced, and treated for 4 hours with 4 mg/ml collagenase (type I; Worthington Biochemical, Freehold, NJ) in Dulbecco's modified Eagle's medium (DMEM) at 37°C in 5% CO_2. Dissociated cells were plated in DMEM supplemented with 10% human AB serum (BioWhittaker, Walkersville, MD), 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were used between the third and tenth passages. Where indicated, cells were treated with human recombinant IL-1β (1 ng/ml; Genzyme, Cambridge, MA).

Preparation of cytoplasmic and nuclear extracts. Extracts were prepared using a protocol modified from the method described by Schreiber et al (31). Cells were trypsinized, pelleted, washed in ice-cold phosphate buffered saline, and resuspended in 5 volumes of ice-cold low-salt cytoplasmic buffer (10 m*M* HEPES, pH 7.9 at 4°C, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* dithiothreitol [DTT], and protease inhibitors). Cells were allowed to swell on ice for 15 minutes, after which Triton X-100 was added to a final concentration of 0.25% and cells were vortexed vigorously to disrupt plasma membranes. Intact nuclei were pelleted by centrifugation, and the cytoplasmic extract was aliquoted and immediately frozen. Nuclei were resuspended in high-salt buffer (20 m*M* HEPES, pH 7.9, 400 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, and protease inhibitors) and placed on a shaking platform for 15 minutes at 4°C to disrupt the nuclear membrane. The nuclear extract was collected after centrifugation at 13,500*g* for 5 minutes at 4°C, aliquoted, and immediately frozen. All extracts were stored at −80°C until used.

Western blot analysis. Fifty micrograms of protein from cytoplasmic or nuclear extracts was separated by electrophoresis in a denaturing sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to an activated nylon membrane (PVDF-Plus; Micron Separations, Westborough, MA). Rabbit polyclonal antibodies to the amino terminus of p65 and p105 and the carboxy terminus of c-*rel* were a generous gift from Dr. Nancy Rice (National Cancer Institute, Frederick, MD). Rabbit polyclonal IgG to a synthetic human COX-2 polypeptide, and a COX-2 electrophoresis standard, were purchased from Caymen Chemical Co. (Ann Arbor, MI). Membranes were blocked in 5% (weight/volume) Carnation nonfat dry milk in Tris buffered saline (TBS; 150 m*M* NaCl, 20 m*M* Tris HCl, pH 7.6), primary antibodies were added in fresh blocking solution as indicated and incubated for 1 hour at room temperature, and membranes were washed 3 times in TBS containing 0.1% Tween 20. Membranes were then incubated for 1 hour at room temperature with horseradish peroxidase–conjugated anti-rabbit immunoglobulin diluted 1:5,000 in TBS containing 1% bovine serum albumin, then washed as above. The ECL (enhanced chemiluminescence) system (Amersham, Arlington Heights, IL) was used for detection.

Electrophoretic mobility shift assay. Complementary oligonucleotides with sequences derived from the human COX-2 promoter region with homology to consensus NF-κB binding sites were synthesized. The oligonucleotide from −455 to −427 contained the potential NF-κB site located at −446 to −437 (5′-CGGCGGGAGAGGGGATTCCCTGCGCC CCCG-3′), and the oligonucleotide from −231 to −202 contained the potential NF-κB site located at −221 to −212 (5′-CAGGAGAGTGGGACTACCCCCTCTGCTCC-3′). An oligonucleotide containing a well-characterized NF-κB consensus site from the human HLA–A3 gene (5′-CCTTGGGGATTCCCCAACTCC-3′) (32) and an oligonucleotide containing a consensus SP-1 binding site (5′-ATTCGATCGGGGCGGGGCGAGC-3′) were used in competition experiments. The underlined sequences delineate consensus binding sites within the synthetic oligonucleotides. Complementary strands were synthesized with 5′ overhanging ends, annealed, and double-stranded oligonucleotides were labeled with ^{32}P-dCTP using the *Klenow* fragment of DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Nu-

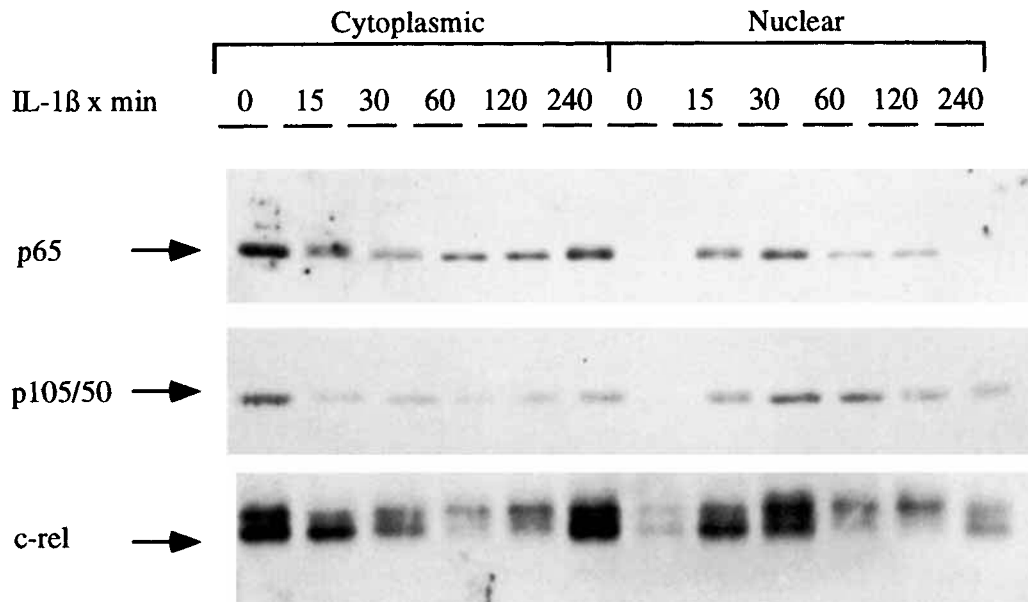
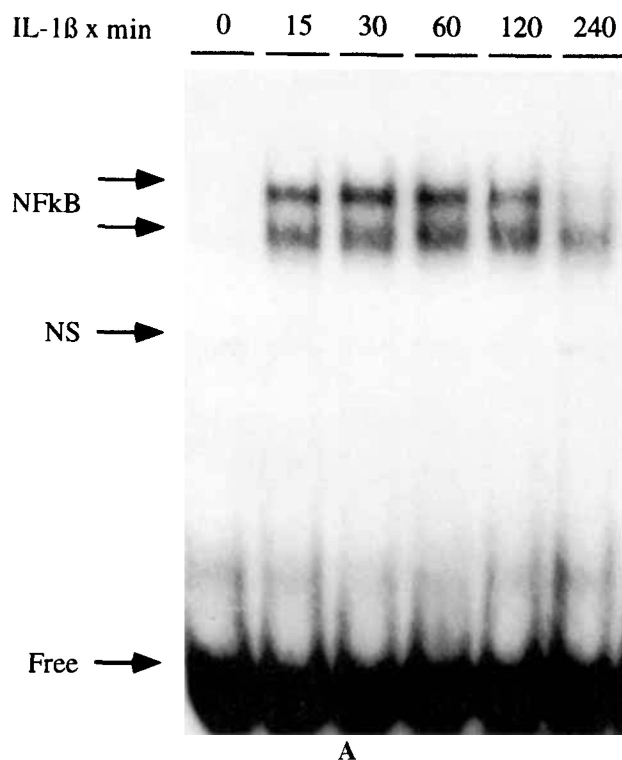


Figure 1. Western blot analysis of nuclear factor κB (NF-κB) subunits in cytoplasmic and nuclear extracts after stimulation of rheumatoid synoviocytes with interleukin-1β (IL-1β; 1 ng/ml). Synoviocytes were used between the third and tenth passages. Cells were treated for the indicated number of minutes (min), after which cytoplasmic and nuclear extracts were prepared as described in Materials and Methods. Proteins (50 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blotting was then performed using antisera to NF-κB family members as indicated. The experiment was performed 3 times with similar findings; a representative result is shown.

clear extracts (5 μg protein) were incubated with 2 μg poly(dI-dC) (Pharmacia, Piscataway, NJ) for 5 minutes in a buffer containing 100 m*M* HEPES, 100 m*M* KCl, 13 m*M* $MgCl_2$, 1 m*M* EDTA, 2.5 m*M* DTT, 1% glycerol, and 2% Ficoll 400. Radiolabeled probe (20,000 counts per minute) was added with or without unlabeled competitor as indicated, and the binding reaction was allowed to proceed for 15 minutes on ice and 15 minutes at room temperature. Reaction mixtures were loaded directly on nondenaturing 4% polyacrylamide gels containing 0.0225*M* Tris-borate and 0.5 m*M* EDTA, and electrophoresed with buffer recirculation for 90 minutes. Gels were dried under vacuum followed by autoradiography. "Supershift" assays were performed as above, except that nuclear extracts were incubated together with antibodies to p65, p105/50, or c-*rel* (described above) for 1 hour prior to addition of the probe.

Reverse transcription–polymerase chain reaction. Total RNA was prepared using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Complementary DNA (cDNA) was prepared by reverse transcription of 5 μg total RNA in 50 μl containing 50 m*M* Tris HCl (pH 8.3), 40 m*M* KCl, 6 m*M* $MgCl_2$, 1 m*M* DTT, 0.4 m*M* dNTPs, 2 μ*M* random hexamer primers (Perkin-Elmer Cetus, Norwalk, CT), 0.2 units/μl RNase inhibitor (Perkin-Elmer Cetus), and 8 units/μl M-MLV reverse transcriptase (Life Technologies, Bethesda, MD). Reaction mixtures were incubated at room temperature for 10 minutes, at 42°C for 30 minutes, and at 95°C for 5 minutes. Complementary DNA was diluted 10-fold, and used for each PCR amplification. PCRs were performed in 50 μl containing 5 μl cDNA, 10 m*M* Tris HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 50 μ*M* dNTPs, [α-^{32}P]dCTP (3,000 Ci/mmole; Amersham), and 0.025 μl *Taq* polymerase (Perkin-Elmer Cetus). The oligonucleotide primers used were as follows: for COX-2, sense 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, antisense 5′-AGATCATCTCTGCCTGAGTATCTT-3′; for G3PDH, sense 5′-CCACCCATGGCAAATTCCATGGCA-3′, antisense 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. Cycling conditions were as follows: denaturing at 95°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 1 minute (25 cycles for G3PDH and 35 cycles for COX-2).

Northern blot analysis. RNA was prepared as described above. Twenty micrograms of total RNA was subjected to electrophoresis through a 1.5% agarose/4% formaldehyde gel, and transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH). A probe was generated using a 1.8-kb fragment of the human COX-2 coding sequence by the random prime method (Prime-It RmT; Stratagene, La Jolla, CA), or from a cDNA fragment of human G3PDH generated by PCR using primers as described above. Membranes were hybridized in 50% formamide at 42°C for 24 hours with 2–5 × 10^6 cpm/ml cDNA probe. Washing was performed twice for 15 minutes in 2× saline–sodium citrate (SSC), 0.1% SDS at room temperature, and twice for 15 minutes in 0.1× SSC, 0.1% SDS at 65°C. The membranes were exposed to Kodak XAR film for up to 2 weeks at −80°C with intensifying screens. Densitometry was performed using a scanner from Molecular Dynamics (Sunnyvale, CA). COX-2 expression was normalized to G3PDH detected on the same Northern blot, for quantitation.



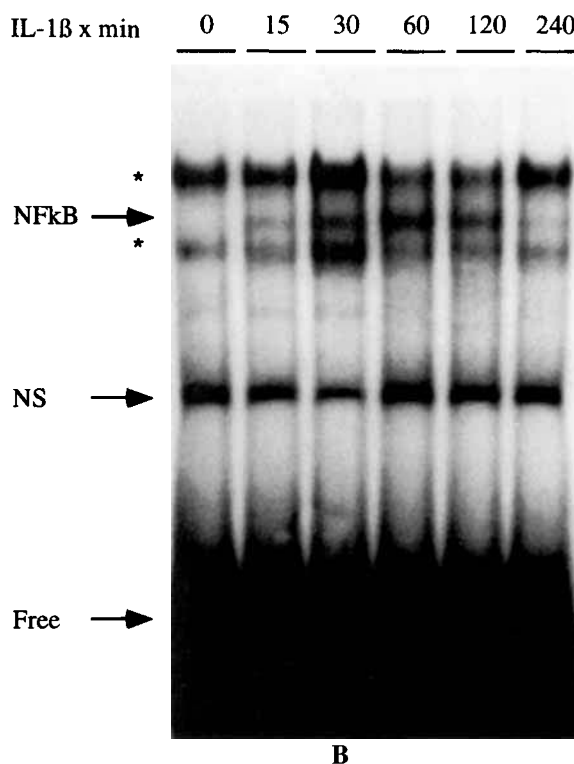


Figure 2. Electrophoretic mobility shift assays demonstrating binding to *cis* sequences of the human cyclooxygenase-2 (Cox-2) promoter/enhancer with homology to nuclear factor κB (NFκB) binding sites after stimulation of rheumatoid synoviocytes with interleukin-1β (IL-1β; 1 ng/ml). **A,** A 30-basepair end-labeled double-stranded oligonucleotide centered on the −446 to −437 NFκB site was used as the probe. **B,** The 30-bp probe contains the NFκB sequence from −221 to −212. Asterisks indicate bands that were not significantly affected by treatment of the cells with IL-1β, and were not NFκB. NS = nonspecific complex; Free = free double-stranded oligonucleotide probe; min = minutes. The experiments were performed 3 times; representative results are shown.

Antisense oligonucleotides. Phosphorothioate-modified sense and antisense oligonucleotides corresponding to the 5′ end of the mRNA for the human p65 subunit of NF-κB and extending 3 nucleotides upstream of the initiation codon were synthesized (Applied Biosystems, Foster City, CA). The antisense oligonucleotide was previously shown by Sokoloski and colleagues to exert biologic effects (33). Sequences were as follows: p65 sense, 5′-GCCATGGACGAA-CCTGTTCCCC-3′; p65 antisense 5′-GGGGAACAGTTC-GGTCCATGGC-3′ (33).

RESULTS

Findings of Western blot analysis of NF-κB. Western blotting of rheumatoid synoviocyte cytoplasmic and nuclear extracts (Figure 1) revealed that at baseline, rheumatoid synoviocytes contained p65, p105/50, and c-*rel* in the cytoplasm. Constitutive nuclear localization of these NF-κB subunits was variable, but much lower than cytoplasmic levels, if detectable at all. After treatment with IL-1β (1 ng/ml), translocation of p65, p105/50, and c-*rel* from the cytoplasm to the nuclear compartment occurred within 15 minutes, was maximal by 30 minutes, and began to decline by 1 hour after treatment. By 4 hours after treatment with IL-1β, the levels of these NF-κB family members in the nucleus and cytoplasm had returned to near baseline levels.

Findings of electrophoretic mobility shift assays. The time course of binding to the potential NF-κB sites derived from the human COX-2 promoter is shown in Figures 2A and B. Using the probe containing either the −446 to −437 or the −221 to −212 sequences, we demonstrated increased binding within 15 minutes after treatment with IL-1β; this reached maximal levels by 30 minutes to 1 hour, then began to decline by 2–4 hours. It should be noted that there were 2 additional bands not

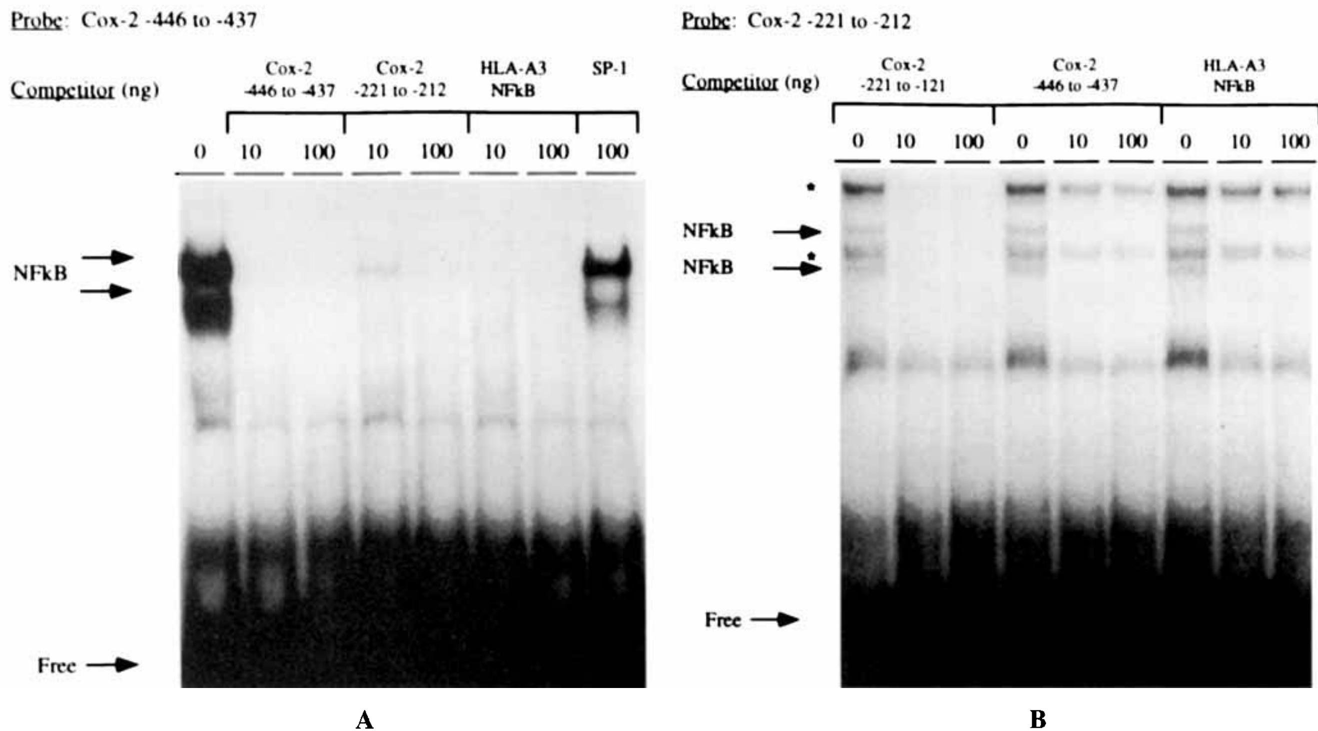


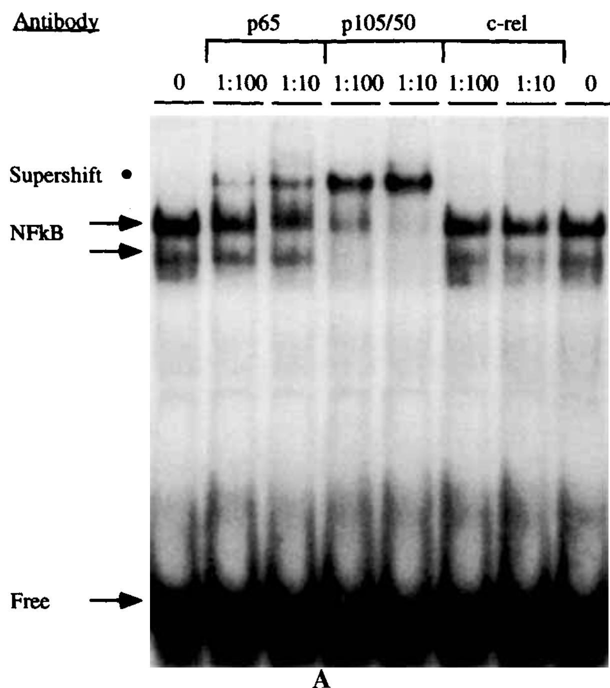
Figure 3. Electrophoretic mobility shift competition assays demonstrating specificity of binding to the NFκB sites of the Cox-2 promoter/enhancer. **A,** The probe contained the −446 to −437 NFκB site from human Cox-2, and unlabeled competitor is as indicated. The HLA-A3–derived competitor was a 21-basepair sequence containing a consensus NFκB binding site, and the SP-1 competitor contained the binding site for that transcription factor which is unrelated to NFκB. **B,** The probe contained the −221 to −212 NFκB binding site. Cold competitor was as indicated. Asterisks indicate bands that were not decreased by the Cox −446 to −437 or HLA-A3 NFκB binding sites. See Figure 2 for definitions.

significantly regulated by IL-1β that were present when the probe containing the −221 to −212 sequence was used. The identity of the proteins in these complexes, and their significance, is not known.

Competition electrophoretic mobility shift experiments using the probe containing the −446 to −437 NF-κB site (Figure 3A) revealed 2 complexes that were specifically competed away by unlabeled oligonucleotides containing the −447 to −436 COX-2 NF-κB site, the −221 to −212 COX-2 NF-κB site, and the HLA-A3 NF-κB site. The double-stranded oligonucleotide containing the consensus SP-1 binding site did not compete significantly with the labeled probe. Competition experiments using the labeled probe containing the −221 to −212 COX-2 NF-κB site (Figure 3B) demonstrated 2 complexes that were competed away by all unlabeled double-stranded oligonucleotides containing NF-κB sites. Two additional complexes that were not significantly diminished by NF-κB oligonucleotides were seen. These are the same complexes that were not significantly affected by treatment with IL-1β.

We used "supershift assays," in which extracts were preincubated with antibodies to the p65, p105/50, and c-*rel* NF-κB family members prior to electrophoretic mobility shift assays, to evaluate the composition of the complexes binding to the COX-2 NF-κB sites (Figures 4A and B). We determined that the upper complex, detected using labeled probes containing either of the COX-2 NF-κB binding sites, contained a p65–p50 heterodimer. The lower complex seen in Figure 4A comprised a p50 homodimer. A barely detectable "supershift" complex was present after preincubation with the anti–c-*rel* antibody, leaving open the possibility that a c-*rel*–containing heterodimer migrating with the same properties of the other complex(es) also binds to the human COX-2 promoter NF-κB sites.

Time course of IL-1β–stimulated COX-2 expression. The time course for induction of endogenous COX-2 mRNA by IL-1β (1 ng/ml) was investigated using RT-PCR and Northern blotting. Compared with levels of the housekeeping gene, G3PDH, there was a rapid rise of endogenous COX-2 message by 30 minutes after stimulation, which peaked at 1 hour and began to diminish by 2 hours. A second rise in COX-2 mRNA



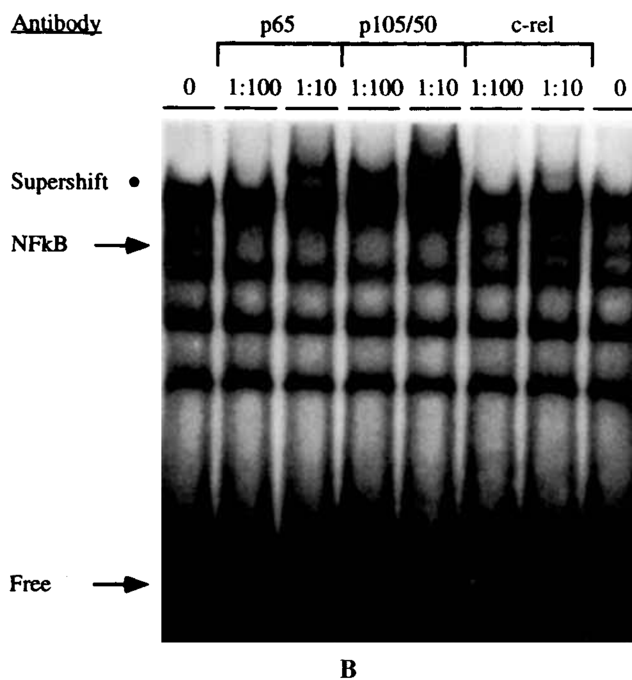


Figure 4. Electrophoretic mobility shift competition "supershift" assays identifying NFκB family members that bind to the Cox-2 promoter. **A,** Using the probe containing the −446 to −437 sequence, the p65–p50 heterodimer was identified as forming the upper complex, since preincubation with antisera to both these subunits yielded a supershift (**asterisk**) and a decrease in the intensity of the upper band. The lower band was formed by binding of a p50 homodimer, as indicated by loss of the lower complex using antisera to the p50 subunit alone. A faint band was detected with the higher concentration of antisera to c-*rel*, possibly indicating that a complex containing c-*rel* may bind to this site. **B,** Using the probe containing the −221 to −212 NFκB site, binding of a p65–p50 heterodimer was demonstrated. Several non-NFκB bands are present (see Figures 2B and 3B). See Figure 2 for definitions.

expression was seen at 4 hours (Figures 5A and B). The RT-PCR was performed 4 times with similar results. Northern blotting confirmed the RT-PCR result.

COX-2 protein levels had increased by 1 hour, and expression continued to be increased at 24 hours after treatment (Figure 6).

Response to treatment with NF-κB p65 antisense oligonucleotide. To determine the functional effect of NF-κB binding on induction of COX-2 mRNA, we pretreated RA synoviocytes with phosphorothioate-modified antisense oligonucleotides corresponding to the 5′ end of the p65 mRNA and including 3 nucleotides upstream of the initiation codon (40 μM) or sense oligonucleotides (40 μM) as negative controls. Binding of the p65–p50 heterodimer to the COX-2 promoter was markedly diminished in the antisense-treated synoviocytes by 4 hours after IL-1β treatment (Figure 7). We further demonstrated that the p65 antisense phosphorothioate oligonucleotides diminished expression of IL-1β–stimulated COX-2 polypeptide at 4 hours after treatment (Figure 8). There are 2 probable reasons that COX-2 polypeptide expression was not decreased to the same extent as NF-κB binding at the 4-hour time point. First, use of antisense technology prevents only synthesis of new p65, not translocation of preformed cytoplasmic p65. Therefore, no change in early NF-κB binding to the COX-2 promoter after treatment with the p65 antisense oligonucleotides would be expected. Transcription could be increased through NF-κB–dependent mechanisms prior to new synthesis of p65, leading to some increase of COX-2 polypeptide expression. Second, as outlined below, there are multiple transcription factors that may stimulate transcription of COX-2.

DISCUSSION

PGs mediate both inflammatory and destructive features of RA. The classic signs of acute inflammation

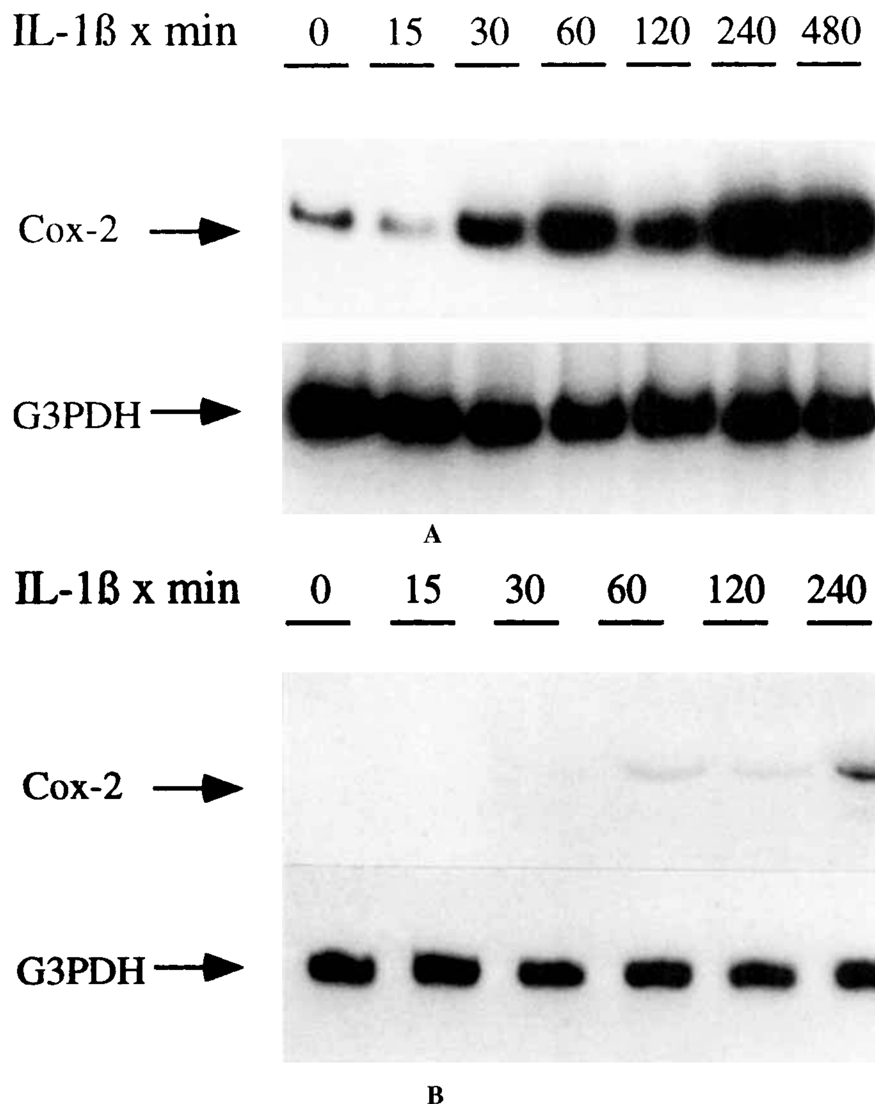


Figure 5. Time course of increased Cox-2 messenger RNA (mRNA) expression in rheumatoid synoviocytes after stimulation with IL-1β (1 ng/ml), in studies using **A,** reverse transcriptase–polymerase chain reaction (RT-PCR) and **B,** Northern blot analysis. Increased Cox-2 expression is shown with G3PDH mRNA expression from the same RT samples or by reprobing the same filter (shown for comparison). No bands were present when PCR was performed with no RT or no RNA in the RT reactions. See Figure 2 for other definitions.

(pain, swelling, erythema, and warmth), can be reproduced by PG actions which stimulate vasodilatation, increase vascular permeability, and contribute to nociceptor sensitization (34). PGE_2 contributes to increased expression of matrix metalloproteinases that are important mediators of tissue degradation (3,5,6,35). In addition, PGE_2 stimulates production of vascular endothelial growth factor by synoviocytes that may contribute to angiogenesis (7). It is likely that increased local PG production during inflammation is stimulated by interactions between resident cells and infiltrating mononuclear cells. These mononuclear cells probably contribute to increased PG production by mechanisms including direct cell–cell contact and, in particular, elaboration of cytokines (16,35). Regulation of PG production in RA involves multiple enzymatic steps, and may differ depending on cell type. For example, IL-1 increases secretory group II phospholipase A_2 ($sPLA_2$), but not cytosolic PLA_2 ($cPLA_2$), in chondrocytes (35), while stimulating $cPLA_2$,but not $sPLA_2$, in synoviocytes

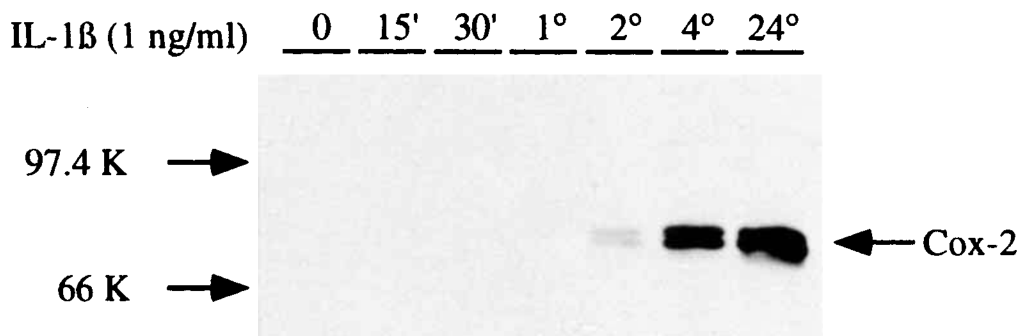


Figure 6. Western blot demonstrating the time course of increased Cox-2 polypeptide expression in rheumatoid synoviocytes after stimulation with IL-1β (1 ng/ml). Cox-2 expression was not detectable at baseline, but was increased by 2 hours (°). Expression continued to increase over the 24-hour time period. The typical doublet represents differently glycosylated forms of Cox-2 and migrates at ~72 kd. Migration of protein standards is indicated on the left. ′ = minutes; see Figure 2 for other definitions.

(17). In all cell types within joint tissues that have been examined, COX-2 expression is dramatically increased by IL-1 (16,17,37–39). Regulation of COX-2 expression is likely a critical step in determining levels of PG produced in the joint during inflammation (40).

We demonstrated that IL-1β increases translocation of NF-κB subunits to the nucleus in rheumatoid synoviocytes. The cells used in these experiments were predominantly type B synoviocytes, based on our previous experience that synovial cells prepared in this way express only very low levels of CD11b/CD18 (MO1) and CD14 (MO2) (41). Fujisawa and colleagues recently demonstrated that tumor necrosis factor α (TNFα) also increases activation of NF-κB in rheumatoid synovial cells (42). We have also shown that TNFα stimulates translocation of NF-κB to the nucleus (data not shown).

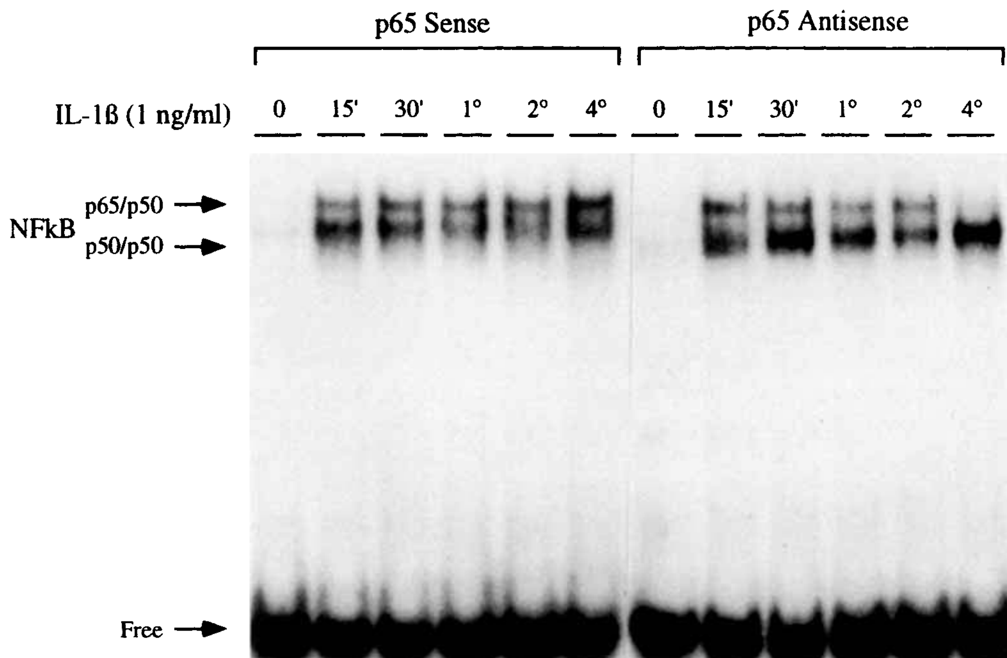


Figure 7. Electrophoretic mobility shift assay demonstrating loss of the p65/p50 band 4 hours (°) after treatment with antisense oligonucleotide to p65 (40 μM). The probe used contained the −446 to −437 NFκB site. There was no effect on binding when the p65 sense oligonucleotide was used. ′ = minutes; see Figure 2 for other definitions.

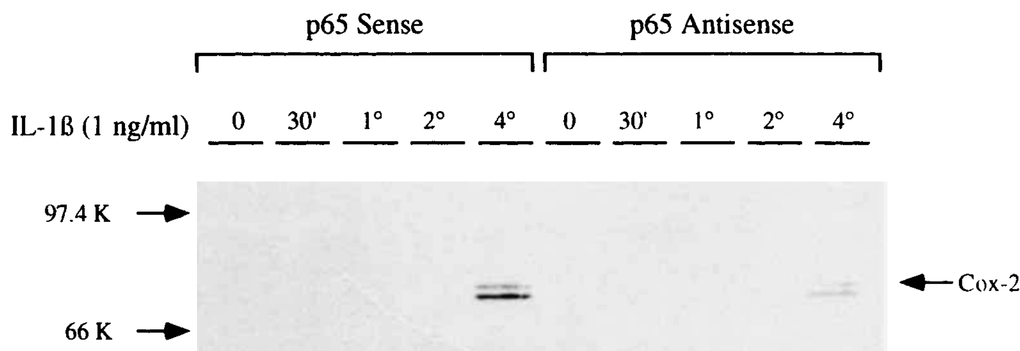


Figure 8. Western blot demonstrating decreased Cox-2 expression after stimulation with IL-1β (1 ng/ml) in synoviocytes pretreated with antisense oligonucleotide to p65 (40 μM). The typical doublet represents differently glycosylated forms of Cox-2 and migrates at ~72 kd. Migration of protein standards is indicated on the left. ′ = minutes; ° = hours; see Figure 2 for other definitions.

In addition, TNFα increases NF-κB binding to the COX-2 promoter, COX-2 mRNA expression, and polypeptide expression (data not shown). These data suggest that both IL-1β and TNFα may use the NF-κB pathway to stimulate COX-2 expression in rheumatoid synoviocytes.

Constitutive nuclear NF-κB subunit expression in the established primary rheumatoid synoviocyte cell lines used in our experiments was variable, but usually quite low. However, it is of interest that Okamoto and colleagues reported that freshly isolated rheumatoid synovial cells constitutively express nuclear NF-κB (43), and Marok and coworkers demonstrated expression of an active form of NF-κB in vivo in RA synovia (44). The latter investigators used antisera specific for the nuclear localization sequences of the Rel-A (p65) subunit of NF-κB, unmasked only after dissociation from IκB and therefore presumed to represent "active" subunit, to perform immunohistochemical analysis of RA synovia. They demonstrated constitutive nuclear expression of Rel-A (p65), predominantly localized to the blood vessels in patients with an acute disease flare at the time of tissue sampling, but also in the synovial lining containing predominantly type A synoviocytes and subsynovia where type B synoviocytes are localized (44). These findings may be explained, at least in part, by persistent COX-2 expression in vivo in patients with RA, since immunohistochemical staining with specific anti–COX-2 antisera was also localized predominantly to the blood vessel endothelial cells (16).

In parallel with IL-1–stimulated nuclear translocation of NF-κB, we demonstrated induction of specific NF-κB binding to 2 *cis* sequences in the human COX-2 promoter. IL-1β–stimulated increases in COX-2 mRNA and polypeptide expression occurred after an appropriate lag time after NF-κB binding. Inhibition of NF-κB binding by pretreatment with phosphorothioate-modified p65 antisense oligonucleotides led to diminished COX-2 protein expression. These data demonstrate that inhibition of a transcription factor can directly affect expression of endogenous COX-2.

Investigation of NF-κB regulation of COX-2 transcription is of interest in light of recent data suggesting that one mechanism by which glucocorticoids exert down-regulatory effects may be by inhibition of NF-κB translocation to the nucleus. Although glucocorticoids, through interaction with the glucocorticoid receptor (GR), can function by binding directly to the glucocorticoid response element (GRE) in promoter regions to mediate effects on gene transcription, there are no GREs in most immune response genes, including COX-2 (30). In addition, although glucocorticoids also act to inhibit transcription via physical association between the GR and activating protein-1 (AP-1), no AP-1 site has been identified as yet in the human COX-2 promoter. It was, however, recently reported that glucocorticoids enhance expression of IκB, which results in decreased nuclear localization and binding of NF-κB (45,46). This may occur either through "recapture" of NF-κB in the cytoplasm prior to nuclear translocation, or translocation of newly synthesized IκB to the nucleus, where it can promote dissociation of DNA-bound NF-κB (45,46).

While NF-κB is one mechanism by which IL-1 may stimulate COX-2 expression, biologic effects of IL-1 are complex and relatively poorly understood. Many of the cellular responses to binding of the IL-1 ligand to IL-1RI take place very rapidly (within 15 minutes),

including translocation of transcription factors to the nucleus, as we demonstrated. After ligand binding, the complex is rapidly internalized, with some complexes degraded and others found associated with the nucleus. However, it has been demonstrated that late displacement of IL-1 from the type I receptor blocks transcription, suggesting that the continued presence of IL-1 on the IL-1RI is critical to amplification of IL-1–mediated biologic effects (26). While most intracellular effects of IL-1 occur rapidly and are short-lived, at least one potential mediator of IL-1 activity with sustained activity was described by Guesdon and coworkers, who demonstrated the persistent presence of active β-casein kinase (47). Transcriptional results mediated by this pathway have not yet been fully defined.

Multiple transcription factors, including not only NF-κB, but also nuclear factor for IL-6 (NF-IL6, also known as the CCATT enhancer binding protein or c/EBPδ) and the *c-fos* and *c-jun* components of AP-1, are activated in response to IL-1 in various cell systems (26). Binding sites for NF-IL6 (c/EBPδ) are present in the COX-2 promoter. Using constructs containing the human COX-2 promoter linked to a luciferase reporter gene, Inoue and coworkers demonstrated that c/EBPδ was involved in transcriptional regulation of human COX-2 in bovine and human endothelial cells, and that binding of this transcription factor to both the NF-IL6 and CRE binding sites probably occurred (48). Using similar methodology, that group also reported a functional requirement for the CRE element of the human COX-2 promoter/enhancer for COX-2 expression during differentiation of U937 monocytic cells (49).

IL-1 may also mediate posttranscriptional regulation of COX-2 expression. COX-2 transcripts exhibit a very high turnover rate, likely mediated by multiple AU-rich sequences located in the 3′-untranslated region of the mRNA (37). These sequences are present in a number of lymphokine and protooncogene mRNAs, and accelerate mRNA degradation (50). Rïstimaki and colleagues demonstrated that the half-life of COX-2 mRNA in an immortalized human umbilical vein endothelial cell line was approximately 1 hour. Treatment with IL-1 increased the stability of the COX-2 transcript, an effect that was enhanced by a transcription inhibitor (37). These data support the possibility of a short-lived factor(s) inhibited by IL-1 that degrades COX-2 mRNA.

Our data and data from other groups support the concept of multiple mechanisms underlying the effects of IL-1 on COX-2 expression. We demonstrated that NF-κB is involved in IL-1–stimulated COX-2 mRNA expression. NF-IL6 (c/EBPδ) involvement in transcriptional regulation of COX-2 remains to be evaluated in synoviocytes, but clearly plays a role in expression of the human COX-2 gene in other cell types (48). Increased mRNA stability mediated by IL-1 may be another mechanism likely to be important in increased COX-2 expression (37). Finally, the contribution of glucocorticoid effects on NF-κB nuclear translocation to inhibition of COX-2 expression and PG production must be evaluated. Understanding the molecular mechanisms underlying increased and sustained COX-2 protein expression and PG production in rheumatoid synovial tissues may help to elucidate factors responsible for chronic inflammation and tissue destruction in RA.

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