Manipulation of Distinct NFκB Proteins Alters Interleukin-1β-induced Human Rheumatoid Synovial Fibroblast Prostaglandin E₂ Formation

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Interleukin 1β (IL-1β) up-regulates human rheumatoid synovial fibroblast (RSF) 85-kDa phospholipase A₂ (PLA₂) and mitogen-inducible cyclooxygenase (COX) II. Promoter regions for these genes contain a motif that closely resembles the “classic” NFκB consensus site. Immunoblot analysis identified NFκB1 (p50), RelA (p65), and c-Rel in RSF. Upon IL-1β-stimulation, p65 and c-Rel but not p50 protein levels were reduced suggesting nuclear translocation. IL-1β-induced RSF nuclear extracts contained a p65-containing complex, which bound to the classical NFκB consensus motif. An NFκB classical oligonucleotide decoy produced a concentration-dependent decrease in IL-1-stimulated PGE₂ production (IC₅₀ = −2 μM), indicating a role of NFκB. Utilization of antisense technology showed that p65 but not p50 or c-Rel mediated IL-1β-stimulated PLA₂ formation. Treated RSF could not transcribe COX II or 85-kDa PLA₂ mRNA, which reduced their respective proteins. Interestingly, stimulated IL-8 production was not inhibited by the classical NFκB decoy but was reduced by treatment with antisense to both p65 and c-Rel supporting preferential binding of c-Rel-p65 to the “alternative” IL-8 xB motif. Taken together, these data provide the first direct evidence for a role of p65 in COX II and 85-kDa PLA₂ gene induction and support the IL-1 activation and participation of distinct NFκB protein dimers in RSF prostanoid and IL-8 formation.

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation and hyperproliferation of the synovial lining (1). Enhanced levels of the cytokine, interleukin (IL)-1β, perpetuate the disease process through up-regulation of a multitude of factors leading to eicosanoid formation, matrix degradation, bone resorption, and proliferation in the joint (2–6). We and others have demonstrated that human rheumatoid synovial fibroblast (RSF) prostaglandin (PG) E₂ accumulation in response to IL-1β is a direct result of the coordinate up-regulation of 85-kDa phospholipase A₂ (PLA₂) and the induction of COX II (6–8). Indeed, we reported that depletion of IL-1β-induced 85-kDa PLA₂ to basal levels by antisense severely compromised the ability of RSF to make PGE₂. However, the mechanism(s) by which IL-1β regulates 85-kDa PLA₂ and COX II gene induction in this system have not been elucidated.

IL-1β is a potent activator of nuclear factor κB (NFκB) (1, 9, 10) in other cell systems, and this transcription factor in turn regulates a wide variety of inflammatory and immunoregulatory genes (10–16). 5′-flanking regulatory regions for both the human 85-kDa PLA₂ and COX II genes have recently been isolated (17, 18), and sequence analysis has identified a number of possible transcription factor consensus binding motifs, including NFκB. The putative NFκB motif in the 85-kDa PLA₂ promoter is located at −1099 base pairs (17), whereas the NFκB consensus site in the human COX II promoter is located at −233 base pairs (18).

NFκB is a dimeric DNA binding protein comprised of members of the NFκB/Rel/dorsal family of proteins including the mammalian forms, NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB, and c-Rel (10, 11). NFκB proteins are capable of forming numerous homodimers and heterodimers with other family members, and this conveys a degree of NFκB gene target specificity. In addition, the different dimeric pairs bind to different NFκB DNA motifs with varying affinities, again permitting specificity (19, 20). These motifs can be subdivided into two main categories, the “classic” xB consensus site (GGGRN-NTYCC) and the “alternative” or xB-like motif (T/AC/GGAR-NYCC). Reports demonstrated that different homodimer or heterodimer pairs exhibit varied affinity for these two motif classes. The p50-p65 heterodimer has been shown to bind preferentially to the decameric oligonucleotide sequence (GGACTTTCC) present in Ig κ-light chain enhancer (20). The NFκB motifs present in the 85-kDa PLA₂ (GGGAAATTCCTC) and COX II (GGGACTACC) promoters most closely resemble classic κB consensus motifs. In contrast, the alternative motif, present in promoters such as IL-8, intercellular adhesion molecule, and tissue factor, binds c-Rel-p65 heterodimers with higher affinity than p50-p65 (20).

Double-stranded NFκB decoys are an effective approach to specifically modulate expression of NFκB-driven reporter genes through successful competition of the decoy with the authentic DNA motifs, preventing dimer translocation and/or DNA binding (21). In addition, antisense technology has been successfully used to modulate NFκB proteins to assess their role in the transcriptional control of a variety of genes, such as the elucidation of a role for p65 but not p50 in IL-8 expression (14). Herein, we demonstrate that IL-1β induces the activation of NFκB in human RSF and that modulation of NFκB activation using the tools described above interferes with prostanoid
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MATERIALS AND METHODS

RSF Culture and Eicosanoid Measurement—Primary cultures of human RSF were obtained from 10 adult patients and maintained in culture as described previously (6, 22). For some studies, fibroblasts were plated at 5 × 10⁴ cells/ml in 16-mm (diameter) 24-well plates (Costar, Cambridge, MA) and used when confluent. In studies using antisense, the medium containing 10% fetal bovine serum was removed and serum-free medium supplemented with inulin, transferrin, and sodium selenite (Sigma) was added. Cells were exposed to IL-1β (1 mg/ml, Upstate Biotechnology, MA) for the designated time, after which PGE2 levels or IL-8 levels in cell-free medium were measured using enzyme immunoassay purchased (Cayman Chemical Co., Ann Arbor, MI and Biosource International, Camarillo, CA) as described previously (6). In some studies cells were first treated with NFκB decoys (4 h at 37°C) or antisense (18 h at 37°C) prior to the addition of IL-1β for 0–24 h.

Preparation of Phosphorothioate Oligonucleotides—Initiation site-directed antisense to p65 (SK68), 5′-ggggaaaggctggctccaggg-3′; p50 (SB70), 5′-gggtcgggtcttcggtcctg-3′; c-Rel (SB7221), 5′-taaaggccaggtctcggggttg-3′; control oligonucleotide p50 sense (SB71), 5′-gagtaggcaagatcagacgagtc-3′; and a scrambled oligonucleotide (SB7222), 5′-ttacgcggctgcggcgccgg-3′ (14) were synthesized on a 380A DNA synthesizer from Applied Biosystems using phosphoramidite chemistry as described previously (6, 22). Double-stranded NFκB consensus motifs (decoys) were first synthesized as single-stranded 22-mer phosphorothioate oligonucleotides (NFκB decoy, 5′-agttgaggaggctggctccaggg-3′; mutant decoy, 5′-agttgaggaggctggctccaggg-3′) and then annealed according to standard protocols. Oligonucleotides and decoys were added as a complex with Lipofectin (5 μg/ml of culture medium, Life Technologies Inc.) (6, 22).

RSF Subcellular Fractionation and Immunoblot Analysis—Human RSF were trypsinized, resuspended to 1.0 × 10⁶ cells/ml in homogenization buffer, and disrupted on ice by sonication, and the homogenate was centrifuged at 100,000 × g for 10 min at 4°C to obtain a supernatant (cytosol) and particulate fraction as described previously (6). Cell RSF were trypsinized, resuspended to 1.0 × 10⁶ cells/ml, and nuclei were pelleted by centrifugation at 100,000 × g for 1 h at 4°C, and the nuclear extract, and 0.5 ng of 32P-labeled oligonucleotide probe (~50,000 cpm) with or without unlabeled competitor (20–40-fold excess). The Oct-1 consensus sequence, 5′-tgcgtgaagctcagctagc-3′, was purchased from Santa Cruz Biotechnology, Inc. Reactions were incubated for 20 min at room temperature. In “supershift” studies, 1 μg rabbit anti-p50, anti-p65, or anti-c-Rel serum (Santa Cruz Biotechnology) was added to the reaction and incubated for 45 min at room temperature. Binding reactions were subjected to nondenaturing polyacrylamide electrophoresis through 4% gels in a 1 × Tris-borate-EDTA buffer system. Gels were dried and subjected to autoradiography.

Northern Analysis—Total RNA was isolated from RSF using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Equal concentrations of RNA (20 μg/lane) were subjected to electrophoresis in 1% agarose gel containing formaldehyde. Following electrophoresis, gels were rinsed, transferred to Hybond N+ (Amer sham Corp.) by vacuum blotting (Bio-Rad) in 10 × SSC according to manufacturer’s protocol, and RNA was fixed to the membrane by UV cross-linking (0.12 J/cm²). RNA samples were visualized by staining with 0.02% methylene blue (5 min) and destained in distilled water (15 min). Equal loading was verified by quantitation of ribosomal RNA bands. Hybridizations, following standard prehybridization treatment, were done in prehybridization solution (10 ml/blot) containing 20 ng of denatured specific DNA probe labeled to 1–2 × 10⁹ dpm/μg with 32P (see below) at 68 °C for 18 h. Following hybridization, blots were washed twice with 2 × SSC, 0.1% SDS at 68 °C and then once with 1 × SSC, 0.1% SDS at 68 °C and once with 0.2 × SSC, 0.1% SDS at 68 °C. Filters were analyzed and quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). 0.5 ng of dDNA probe was the full-length cDNA (2.87 kilobases) and the COX II probe was a 1.2-kilobase EcoRI frag of the murine COX II cDNA clone kindly provided by Dr. David DeWitt, Michigan State University. 20 ng of cDNA was labeled using a Rediprime kit and 50 μCi of [32P]dCTP (Amer sham Corp.). Unincorporated nucleotides were removed using Quick Spin columns (Boehringer Mannheim).

Calculations and Statistics—All studies were performed using RSF from two to five different individuals. Data represent mean ± standard deviation (S.D.), n = 3, and subjected to one way analysis of variance and Duncan’s multiple range test (p < 0.05) for statistical evaluation where indicated.

RESULTS

Characterization of IL-1β-induced Activation of NFκB in RSF—Confluent RSF in T-75 flasks were placed in serum-free Eagle’s minimum essential medium for 24 h prior to incubation in the presence of vehicle or IL-1β for 0, 15, or 30 min at 37 °C. Nuclear extracts were prepared and analyzed by EMSA as described under “Materials and Methods.” Fig. 1A shows that a protein complex that binds to the 32P-labeled NFκB classical sequence is present in nuclear extracts of unstimulated and stimulated RSF. IL-1β stimulation of the RSF increased the nuclear levels of the protein complex in a time-dependent manner. The 15-min time point was chosen for subsequent NFκB activation studies because it routinely displayed a 2–3-fold increase in NFκB binding activity as assessed by scanning gel densitometry (area pixel values for Fig. 1: control, 945; IL-1β (15 min), 1909; IL-1β (30 min), 1100: area pixel values for a similar study: control, 1179; IL-1β (15 min), 3773). Specific binding to the NFκB classical motif was demonstrated when labeled probe binding was inhibited by incubation with excess unlabeled NFκB consensus oligonucleotide (20×) but not by an excess of the unrelated Oct-1 oligonucleotide (20×) (Fig. 1B). Use of the COX II αB motif gave identical EMSA results (data not shown). Therefore, the classical motif was used for all subsequent studies.

To determine the presence of p50, p65, or c-Rel in RSF, confluent cells were placed in serum-free medium for 24 h prior to exposure to vehicle or IL-1β (1 ng/ml) for 15 min. After the indicated time, cells were pelleted, and cytosolic fractions or nuclear extracts were prepared using cold lysis buffer. Western analysis of cytosolic fractions (30 μg) confirmed the presence all three NFκB proteins in unstimulated control RSF (Fig. 2). Exposure to IL-1β resulted in 75 and 43% reductions in cyto-

formation and transcriptional up-regulation of 85-kDa PLA2 and COX II.
solic levels of p65 and c-Rel, respectively, as measured by scanning gel densitometry (area pixel values: p65 nontreated control, 591; p65 IL-1 treated, 157; c-Rel nontreated control, 675; c-Rel IL-1 treated, 388). However, stimulation with IL-1 did not alter p50 over this time frame because there was no detectable change in p50 immunoreactive protein levels (p50 untreated control, 864; p50 IL-1 treated, 938).

In order to evaluate the presence of NFκB proteins in control and treated RSF nuclear extracts, supershift EMSA analysis was performed using radiolabeled classic NFκB motif and antisera specific for p50, p65, or c-Rel. Fig. 3 shows one representative supershift EMSA. Exposure of RSF to IL-1 resulted in the appearance of an NFκB binding complex, which could be supershifted by antiserum to p65. Neither antiserum specific for p50 nor that specific for c-Rel induced a supershift in either control or stimulated nuclear extracts using the conditions described in this study. In the case of c-Rel, the lack of binding affinity for c-Rel containing dimers to classical NFκB could also account for the lack of a supershift (20). The possibility that the antibodies recognize epitopes that are concealed by DNA binding cannot be ruled out.

The Effect of NFκB Decoys on IL-1β-induced PGE₂ Formation—Double-stranded oligonucleotide decoys (22-mers) containing the classic NFκB consensus site or a mutant NFκB consensus site were prepared by phosphoramidite chemistry, annealed, and complexed to Lipofectin as described under “Materials and Methods.” RSF in Eagle’s minimum essential medium supplemented with insulin, transferrin, and sodium selenite were incubated with the NFκB decoy (0.3–3.0 μM), the mutant decoy (3.0 μM), or Lipofectin vehicle alone for 4 h at 37°C (21) prior to the addition of IL-1β (18 h, 37°C). Cells were monitored throughout the study, and no greater cytotoxicity was observed in the treated groups compared with the untreated controls as assessed by morphology, adherence, and trypan blue exclusion. Fig. 4A shows that the NFκB decoy but not the mutant decoy concentration-dependently reduced IL-1β-induced PGE₂ formation. Pretreatment of IL-1β-stimulated cells with either 1.0 or 3.0 μM NFκB decoy resulted in a 43% or a 57% reduction in PGE₂ production, whereas preincubation with the mutant decoy failed to attenuate PGE₂ levels. In contrast, IL-1-induced IL-8 production by the same RSF was
not significantly altered by pretreatment with either the NFκB decoy or the mutant decoy (Fig. 4B).

**Effect of NFκB Antisense on IL-1β-induced RSF PGE₂ and IL-8 Formation**—To specifically modulate individual NFκB proteins, fibroblasts in serum-free Eagle’s minimum essential medium were incubated (18 h at 37°C) in the presence of Lipofectin vehicle or increasing concentrations (0.03–0.3 μM) of antisense to p65 (SB68), p50 (SB70), c-Rel (SB7221), or a scrambled oligonucleotide control (SB7222) as described under “Materials and Methods.” After the indicated time, cells were exposed to IL-1β (8 h, 37°C), and cell-free medium was analyzed for PGE₂ levels as described under “Materials and Methods.” Data are expressed as a mean of the percentage of stimulated control level ± S.D. (stimulated PGE₂ range, 3.0–27.7 ng/ml; stimulated IL-8 range 1.1–9.4 ng/ml) of triplicate determinations from three to five donors. * indicates significant differences from IL-1β control at p < 0.05.

**Fig. 5.** NFκB antisense dose-dependently reduces IL-1β-induced RSF PGE₂ and IL-8 formation. Human RSF were exposed to Lipofectin vehicle or increasing amounts of p65 antisense (SB68), p50 antisense (SB70), c-Rel antisense (SB7221), or control oligonucleotide (SB7222) for 18 h prior to 8 h of stimulation with IL-1β (1 ng/ml). Cell-free medium was removed and analyzed for PGE₂ (A) levels or IL-8 levels (B) as described under “Materials and Methods.” Data are expressed as a mean of the percentage of stimulated control level ± S.D. (stimulated PGE₂ range, 3.0–27.7 ng/ml; stimulated IL-8 range 1.1–9.4 ng/ml) of triplicate determinations from three to five donors. * indicates significant differences from IL-1β control at p < 0.05.

E.g. modest nonspecific reductions or potentiations. Nonetheless, all donors consistently responded to p65 antisense and subsequent studies examining the effect of p65 antisense on PGE₂ formation were performed at 0.3 μM.

**Effect of p65 Antisense on COXII and 85-kDa PLA₂**—Fig. 6A shows one of two representative experiments demonstrating that exposure to IL-1β caused the accumulation of COX II immunoreactive protein, whereas little or no COX II was evident in nonstimulated RSF as has been previously reported (6–8). Pretreatment with p65 antisense significantly inhibited the IL-1β-induced increase in COX II immunoreactive protein levels. RSF of this particular donor displayed some sensitivity to nonspecific oligonucleotide treatment as a marginal reduction in COXII protein expression occurred with the control oligonucleotide. This was mirrored in the PGE₂ levels measured in the study (unstimulated PGE₂, 0.4 ng/ml; IL-1β-stimulated PGE₂, 10.1 ng/ml; SB68 + IL-1β PGE₂, 11.1 ng/ml; SB71 + IL-1β PGE₂, 8.9 ng/ml). Western analysis of the 85-kDa PLA₂ revealed no change in protein levels with either p65 antisense or control oligonucleotide after 8 h (data not shown). However, evaluation of samples treated identically but exposed to IL-1β for 24 h rather than 8 h showed that 85-kDa PLA₂ protein was reduced...
Therefore, it is unlikely that this family member is involved in synovial fibroblast IL-1-driven prostanoid formation.

It is well established that many inflammatory inducers such as IL-1β, tumor necrosis factor, and lipopolysaccharide stimulate PGE₂ formation from a wide variety of cell types including monocyte/macrophage, fibroblast, and endothelial cells (29). Further, these ligands are known to mediate the activation of NFκB (30). As such, a role for this transcription factor in the regulation of stimulated prostanooid formation could be envisioned. Little is known about the transcriptional control of either the COX II or the 85-kDa PLA₂ gene expression. The cyclic AMP response element has been shown to be necessary for COX II induction in phorbol ester-differentiated human U937 cells (18), and the importance of both the cyclic AMP response element and NF-IL6 sites for COX II expression in monocyte/macrophage or fibroblast cell lines has been described (31). In a murine osteoblast cell line, NFκB and NF-IL-6 were important in tumor necrosis factor-stimulated PGE₂ production (32). Here we provide evidence for a role of NFκB in IL-1-stimulated PGE₂ formation. This is in line with recent observations that glu-
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corticoids, which inhibit prostanoid formation act, in part, through regulation of NFKB activation (33).

Exposure to the NFKB decoy, mimicking the classical motif, but not the mutant decoy specifically reduced RSF PGE2 levels in response to IL-1. This indicated that dimers displaying high affinity for the classical xB motif were important in IL-1-induced PGE2 formation. Interestingly, IL-1-induced IL-8 production was not affected, which suggested involvement of a distinct NFKB, one that had little or no affinity for the classical motif. This was not unexpected because the IL-8 promoter contains an NFKB element, which represents an alternative xB motif. Because c-Rel-p65 binds to the alternative site, the data could suggest that this heterodimer is not involved in PGE2 regulation.

To specifically delineate which NFKB proteins are involved in PGE2 biosynthesis, NFKB antisense oligonucleotides to p65, p50, and c-Rel were utilized. Antisense has been successfully used to demonstrate specific NFKB regulation of proinflammatory mediators and models of cell adhesion such as CD11b expression in differentiated HL-60 leukemia cells (34) and murine embryonic stem cell adhesion (35). Kusch and Rosen successfully demonstrated that antisense oligonucleotides to p65 but not p50 reduced phorbol myristate acetate-induced Jurkat T lymphocytes IL-8 production (14).

In RSF, utilization of antisense demonstrated the importance of p65 but not p50 or c-Rel in IL-1β-induced PGE2 formation. The lack of effect of p50 antisense in this system could be due to p50 protein stability over the time course of our studies, or it is possible that an alternative heterodimer can compensate for the loss of p50. This particular p50 antisense oligonucleotide was effective in inhibiting cell to substratum adhesion in differentiated embryonic stem cells but not in undifferentiated embryonic stem cells where p65 antisense was shown to be effective (35). The lack of effect of p50 antisense in our system therefore indicates that p50 is not a dimeric partner in the induction of PGE2, c-Rel antisense also had no effect on stimulated PGE2 formation. Alternatively, IL-1-stimulated IL-8 production was reduced by antisense to both p65 and c-Rel consistent with the preferential binding of a c-Rel-p65 heterodimer to the IL-8 xB motif and the lack of affinity of the classical xB motif for c-Rel containing dimers. This strongly suggests that c-Rel is not the dimeric partner participating with p65 in the regulation of COX II and 85-kDa PLA2 gene expression.

The reduced capability of p65 antisense-treated RSF to produce PGE2 correlated with a reduction in both COX II and 85-kDa PLA2 mRNA levels. At 8 h of IL-1 exposure, a time point where COX II protein is clearly present (6–8), p65 antisense treatment resulted in total depletion of COX II protein. The 85-kDa PLA2 protein depletion required a longer time possibly due to a long protein half-life, as suggested by Hulkower et al. (8), or the involvement of more complex post-transcriptional regulatory events (36, 37). Because of the temporal discrepancy in protein depletion, it would appear that the reduction in PGE2 observed in the first 8 h was primarily due to the lack of COX II. Nonetheless, these data support NFKB participation in the regulation of both COX II and 85-kDa PLA2 expression. Indeed, the recent co-localization of both human genes to the same region of chromosome 1 (1q25) (38, 39) suggests the intriguing possibility for coordinate gene regulation.

To our knowledge, these data provide the first direct evidence for a role of the NFKB protein, p65, in regulation of COX II and 85-kDa PLA2 gene expression. Because both are required for IL-1-induced PGE2 formation in RSF, it appears that NFKB activation plays a regulatory role in PGE2 biosynthesis. Finally, we show that PGE2 formation requires NFKB dimers that appear to be different from those that participate in IL-1-driven IL-8 expression.

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