Prostaglandin E$_2$ Regulates the Level and Stability of Cyclooxygenase-2 mRNA through Activation of p38 Mitogen-activated Protein Kinase in Interleukin-1β-treated Human Synovial Fibroblasts*

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The p38 MAPK mediates transcriptional and post-transcriptional control of cyclooxygenase-2 (COX-2) mRNA following interleukin-1(IL-1)/lipopolysaccharide cellular activation. We explored a positive feedback, prostaglandin E$_2$ (PGE$_2$)-dependent stabilization of COX-2 mRNA mediated by the p38 MAPK cascade in IL-1β-stimulated human synovial fibroblasts. We observed a rapid (5 min), massive (>30-fold), and sustained (>48 h) increase in COX-2 mRNA, protein, and PGE$_2$ release following a recombinant human (rh) IL-1β signal that was inhibited by NS-398, a COX-2 inhibitor, and SB202190, a selective, cell-permeable p38 MAPK inhibitor. PGE$_2$ completely reversed NS-398-mediated inhibition but not SB202190-dependent inhibition. The eicosanoid didn't potentiate IL-1β-induced COX-2 expression nor did it activate *COX-2* gene expression in quiescent cells. Transfection experiments with a human COX-2 promoter construct revealed a minor element of p38 MAPK-dependent transcriptional control after IL-1β stimulation. p38 MAPK synergized with the cAMP/cAMP-dependent protein kinase cascade to transactivate the COX-2 promoter. When human synovial fibroblasts were activated with rhIL-1β for 3–4 h (steady state) followed by washout, the elevated levels of COX-2 mRNA declined rapidly (<2 h) to control levels. If PGE$_2$, unlike EP2/3 agonists butaprost and sulprostone, was added to fresh medium, COX-2 mRNA levels remained elevated for up to 16 h. SB202190 or anti-PGE$_2$ monoclonal antibody compromised the stabilization of COX-2 mRNA by PGE$_2$. Deletion analysis using transfected chimeric luciferase-COX-2 mRNA 3′-untranslated region reporter constructs revealed that IL-1β increased reporter gene mRNA stability and translation via AU-containing distal regions of the untranslated region. This response was mediated entirely by a PGE$_2$/p38 MAPK-dependent process. We conclude that the magnitude and duration of the induction of COX-2 mRNA, protein, and PGE$_2$ release by rhIL-1β is primarily the result of PGE$_2$-dependent stabilization of COX-2 mRNA and stimulation of translation, a process involving a positive feedback loop mediated by the EP4 receptor and the downstream kinases p38 MAPK and, perhaps, cAMP-dependent protein kinase.

Cellular activation by external proinflammatory stimuli results in, among other responses, increased phospholipid-derived eicosanoid synthesis that is believed to play a cardinal role in the etiopathogenesis of many immune and inflammatory diseases (1, 2). Additionally, acting locally in an intracrine, autocrine, or paracrine fashion, eicosanoids initiate and modulate cell and tissue responses involved in many physiological processes affecting essentially all organ systems in the human organism (3, 4). Although synthesized through the concerted activity of multiple enzyme systems, the rate-limiting step in the formation of prostanoids is the conversion of arachidonic acid to prostaglandin H$_2$ by cyclooxygenase (COX)[1] (5, 6). A constitutive and inducible form of COX has been identified, and x-ray crystallographic analyses suggest strongly that they are monotopic, endoplasmic reticulum-associated homodimeric enzymes that possess heme-dependent peroxidase and cyclooxygenase activity (7). The constitutive *COX-1* gene has been ascribed a homeostatic function and indeed has a GC-rich housekeeping promoter (8). In contrast, the *COX-2* gene (mRNAs 4.6 and 2.8 kb) is rapidly induced by tumor promoters, growth factors, cytokines, and mitogens in many cell model systems (9–11). It behaves much like an immediate-early gene, and its regulation has been shown to occur at both transcriptional and post-transcriptional levels (12–14). In this regard, the COX-2 message has an extensive 3′-UTR having at least two distinct polyadenylation sites and 22 Shaw-Kamen 5′-AUUU$_n$-A-3′ motifs (11). The latter sequences are believed to be associated with message instability, translational efficiency, and rapid turnover (15, 16). Furthermore, sequence analysis of the 5′-flanking region has shown several potential transcription regulatory sequences, including a TATA box, a c/EBP motif, two AP-2 sites, 3 SP-1 sites, two NF-κB sites, a CRE motif, and an Ets-1 site (no AP-1 site) (11). Nevertheless, despite this wealth of structural information, it is still not totally

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[1] The abbreviations used are: COX, cyclooxygenase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE$_2$, prostaglandin E$_2$; LTB$_4$, leukotriene B$_4$; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; rhIL-1β, recombinant human interleukin-1β; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; ATF-2, activating transcription factor-2; PKA, cAMP-dependent protein kinase; MEK3 or MKK3, p38 MAPK kinase; 3′-UTR, 3′-untranslated region; ARE, AU-rich element; IL-1, interleukin-1; kb, kilobase pairs; TNF-α, tumor necrosis factor-α; HSF, human synovial fibroblasts; PCR, polymerase chain reaction; RT, reverse transcriptase; OA, osteoarthritic; RA, rheumatoid arthritic; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; bp, base pair; NS-398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide]; Luc, luciferase.

clear how the *COX-2* gene is regulated transcriptionally by external stimuli particularly in terms of the relevant signaling pathways and the transcription factors acting on 5′-flanking sequences. Even less is known about post-transcriptional regulation, although it is apparently critical in determining the amplitude and duration of the inductive process.

Interleukin-1 (IL-1) occupies a prominent place in the hierarchy of proinflammatory cytokines associated with inflammatory, immune, and arthritic diseases. Indeed, there is now wide agreement, based on animal models and clinical studies, that the macrophage-derived cytokine plays a fundamental role in the development of osteoarthritis (reviewed in Ref. 17). Among the plethora of genes under IL-1 control, COX-2 is particularly sensitive and is induced rapidly. In many cell types (*e.g.* synovial fibroblasts (18) and endothelial cells (19)), IL-1β induces *COX-2* gene expression by binding to a specific cell-surface receptor (IL-1RI) that has been shown to be the mammalian homolog of the *Drosophila* Toll protein (20). The binding event is followed by the activation of a signaling cascade involving the adapter protein MyD88 that recruits IL-1R-associated kinase and IL-1R-associated kinase 2 to the receptor complex. The latter Ser/Thr kinases interact with the adapter molecule TNF receptor-activated factor-6 that bridges them to the NF-κB-inducing kinase that in turn activates IκB kinases α and β (21–24). With the phosphorylation and degradation of IκBα, NF-κB is released to the nucleus and increases COX-2 promoter activity (25, 26), although this has not been conclusively shown in human synovial fibroblasts. In addition, c/EBP enhancer sequences are also believed to play a role and c/EBPβ/δ synergize transcriptionally with NF-κB for full activation of the human COX-2 promoter (26, 27).

The p38 MAPKs (four isoforms) are members of the MAPK family that are typically activated by environmental stresses and pro-inflammatory cytokines (28). The signal is initiated by membrane-proximal small GTPases of the Rho family, activation of an MAPKKK (*e.g.* MEKK1 and MLK), and phosphorylation and activation of an MAPKK (*e.g.* MKK3/6 or MEK3/6) that in turn phosphorylates and activates p38 kinase (29, 30). p38 kinase can phosphorylate trans-acting factors like ATF-2 and CREB-1 that render them transcriptionally competent (31, 32). An alternative but perhaps less well appreciated mechanism for the mediation of IL-1β signaling is in fact the modulation of p38 MAPK activity (33, 34). It was shown that p38 not only mediates a transcriptional response, presumably at the level of the COX-2 promoter, but also at the level of COX-2 mRNA stability (14, 35, 36). Indeed, the strength and duration of COX-2 expression was largely attributed to the posttranscriptional regulatory phase in which a short (123-nucleotide) fragment of the COX-2 3′-UTR was necessary and sufficient for the regulation of mRNA stability by a p38/MAPKAPK-2/hsp 27 cascade (36). The latter nucleotide sequence interacted with a protein identified as an AU-rich element/poly(U)-binding factor I (36).

In the present study, we report that the magnitude and duration of the induction of COX-2 mRNA, COX-2 protein, and PGE$_2$ release by rhIL-1β is primarily the result of PGE$_2$-dependent stabilization of COX-2 mRNA in primary cultures of human synovial fibroblasts. In addition, PGE$_2$ mitigates COX-2 mRNA decay and inhibition of COX-2 protein translation normally mediated by the 3′-UTR region of COX-2 mRNA. Finally, we provide evidence that the stabilization process involves a positive feedback loop and is mediated by PGE$_2$-dependent up-regulation of the p38 MAPK cascade via the prostaglandin EP4 receptor.

EXPERIMENTAL PROCEDURES

Chemicals—*N*-[2-(Cyclohexyloxy)-4-nitrophenyl)-methanesulfonamide] (NS-398), nimesulide, *N*-[4-nitro-2-phenoxyphenyl)-methanesulfon-amide], indomethacin 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid, prostaglandin E$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$, prostaglandin D$_2$, leukotriene B$_4$, butaprost, and the anti-PGE$_2$ monoclonal anti-body were purchased from Cayman Chemical (Ann Arbor, MI). Crystalline dexamethasone (9-fluoro-11β,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), sodium fluoride, okadaic acid, leupeptin, aprotinin, pepstatin, pyrrolidinedithiocarbamate, phenyl-methylsulfonyl fluoride, actinomycin D, dithiothreitol, and bovine serum albumin were products of Sigma. Verapamil, nifedipine, PD98059, SB202190, KT-5720, and rolipram were purchased from Calbiochem, and Bay 11-7082 was from Biomol (Plymouth Meeting, PA). SDS, acrylamide, bisacrylamide, ammonium persulfate, and Bio-Rad protein reagent originated from Bio-Rad. Tris base, EDTA, MgCl$_2$, CaCl$_2$, chloroform, dimethyl sulfoxide (Me$_2$SO), anhydrous ethanol (95%), methanol (99%), formaldehyde, and formamide were obtained from Fisher. Human recombinant IL-1β (rhIL-1β) and recombinant human tumor necrosis factor-α (TNF-α) were purchased from Genzyme Corp. (Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM), phosphate-free and phenol-red free DMEM, Trizol reagent, heat-inactivated fetal bovine serum, and an antibiotic mixture (10,000 units of penicillin (base), 10,000 μg of streptomycin (base)) were products of Life Technologies, Inc.

Specimen Selection and Synovial Fibroblast Cultures—Synovial lining cells (human synovial fibroblasts, HSF) were isolated from synovial membranes (synovia) obtained at necropsy from donors with no history of arthritic disease (mean age 30 ± 27). Additional experiments were conducted with synovia from osteoarthritic (OA) and rheumatoid arthritic (RA) patients undergoing arthroplasty who were diagnosed based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA/RA (mean age 67 ± 19). Human synovial fibroblasts were released by sequential enzymatic digestion with 1 mg/ml Pronase (Roche Molecular Biochemicals) for 1 h, followed by 6 h with 2 mg/ml collagenase (type IA, Sigma) at 37 °C in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (37, 38). Released HSF were incubated for 1 h at 37 °C in tissue culture flasks (Primaria 3824, Falcon, Lincoln Park, NJ) allowing the adherence of nonfibroblastic cells possibly present in the synovial preparation, particularly from OA and RA synovia. In addition, flow cytometric analysis (Epic II, Coulter, Miami, FL), using the anti-CD14 (fluorescein isothiocyanate) antibody, was conducted to confirm that no monocytes/macrophages were present in the synoviocyte preparation (38, 39). The cells were seeded in tissue culture flasks and cultured until confluence in DMEM supplemented with 10% FCS and antibiotics at 37 °C in a humidified atmosphere of 5% CO$_2$, 95% air. The cells were incubated in fresh medium containing 0.5–1% fetal bovine serum for 24 h before the experiments, and only second or third passaged HSF were used.

Preparation of Cell Extracts and Western Blotting—50–100 μg of cellular extract (in RIPA buffer; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 1 mM NaF) from control and treated HSF were subjected to SDS-polyacrylamide gel electrophoresis through 10% gels (final concentration of acrylamide), under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). Following blocking with 5% BLOTTO for 2 h at room temperature and washing, the membranes were incubated overnight at 4 °C with polyclonal anti-human COX-2 (Cayman Chemical Co., Ann Arbor, MI, 1:7500 dilution) in TTBS containing 0.25% BLOTTO. The second anti-rabbit antibody-horseradish peroxidase conjugate (1:4000 dilution) was subsequently incubated with membranes for 1 h at room temperature, washed extensively for 30–40 min with TTBS, and a final rinse with TBS at room temperature. Following incubation with an ECL chemiluminescence reagent (Amersham Pharmacia Biotech), membranes were prepared for autoradiography, exposed to Kodak X-Omat film, and subjected to digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada, Mississauga, Ontario, Canada) for semi-quantitative measurements. In addition to the anti-COX-2 antiserum, the following polyclonal antibodies were used: total and anti-phospho-p38 MAP kinase (Thr-180/Tyr-182), anti-phospho-ATF-2 (Thr-69/Thr-71), anti-phospho-c-Jun (Ser-63), and anti-phospho-JNK/SAPK (Thr-183/Tyr-85).

Northern Blot Analysis of mRNA—Total cellular RNA was isolated (1 × 10^6 cells = 10–20 μg of RNA) using the Trizol (Life Technologies,

Inc.) reagent. Generally, 5 μg of total RNA were resolved on 1.2% agarose-formaldehyde gel and transferred electrophoretically (30 V overnight at 4 °C) to Hybond-N™ nylon membranes (Amersham Pharmacia Biotech) in 0.5× Tris/acetate/EDTA (TAE) buffer, pH 7. After prehybridization for 24 h, hybridizations were carried out at 50 °C for 24–36 h, followed by high stringency washing at 68 °C in 0.1× SSC, 0.1% SDS. The following probes, labeled with digoxigenin (DIG)-dUTP by random priming, were used for hybridization. Human COX-2 cDNA (1.8 kb, Cayman Chemical Co.) was cloned into the *Eco*RV site of pcDNA 1 (Invitrogen, Carlsbad, CA) and released by *Pst*I and *Xho*I digestion; a 780-bp *Pst*I/*Xba*I fragment was obtained from GAPDH cDNA (1.2 kb; American Type Culture Collection, Manassas, VA). This latter probe served as a control of RNA loading as GAPDH is constitutively expressed. All blots were subjected to a digital imaging system (Alpha G-Imager 2000; Canberra Packard Canada, Mississauga, Ontario, Canada) for semi-quantitative measurements, and changes in COX-2 expression were always considered as a ratio, COX-2/GAPDH mRNA.

Transfection Experiments—Transient transfection experiments were conducted in 4-, 6-, or 12-well cluster plates with HSF as described previously (37). Transfections were conducted using the FuGene 6™ (Roche Molecular Biochemicals) or LipofectAMINE 2000™ reagents (Life Technologies, Inc.) method for 6 h according to the manufacturer's protocol with cells at around 40–50% confluence. HSF were re-exposed to a culture medium + 1% FCS for 2 h prior to the addition of the biological effectors. Transfection efficiencies were controlled by co-transfection with 0.5 μg of pCMV-β-gal, a β-galactosidase reporter vector under the control of cytomegalovirus promoter. The COX-2 promoter (−2390 to +34)-LUC plasmid was kindly provided by Dr. Stephen Prescott, University of Utah (40), as were the chimeric luciferase reporter plasmids fused with the entire human COX-2 mRNA 3′-UTR (1451 bp), AU-rich elements (429 bp of which the first 116 bp contain an AU cluster) or a construct completely devoid of the COX-2 3′-UTR but containing the SV40 poly(A) signal (41). The plasmids are designated LUC-3′-UTR, LUC-+ARE, LUC-Δ3′-UTR. The p38 MAPK kinase (MEK3/MKK3) and pFCPKA$_{cat}$ expression vectors was purchased from Stratagene (La Jolla, CA). Luciferase values, expressed as relative light units, were normalized to 1 OD unit of β-galactosidase activity and protein content per sample for all experiments.

RT-PCR for Luciferase and GAPDH—The oligonucleotide primers for the polymerase chain reactions (PCR) were prepared with the aid of a DNA synthesizer (Cyclone model, Biosearch Inc., Montreal, Quebec, Canada) and used at a final concentration of 200 nmol/liter. The sequences for the luciferase primers are as follows: 5′-ACGGATTAC-CAGGGATTTCAGTC-3′ and 5′-AGGCTCCTCAGAAACAGCTCTTC-3′ (antisense) for the luciferase fragment of 367 bp (41). The sequences for the GAPDH (which served as a standard of quantitation) primers are 5′-CAGAACATCATCCCTGCCTCT-3′, which corresponds to position 604–624 bp of the published sequence, and 5′-GCTTGACAAAGTG-GTCGTTGAG-3′, from position 901–922 bp, for an amplified product of 318 bp (37). Two μg of total RNA, extracted with the Trizol reagent, was reverse-transcribed and then subjected to PCR as described previously (37). RT and PCR assays were carried out with the enzymes and reagents of the GeneAmP RNA PCR kit manufactured by PerkinElmer Life Sciences. Both the RT and PCRs were done in a Gene ATAQ Controller (Amersham Pharmacia Biotech).

The amplification process was conducted over 10–30 cycles in order to define the linear range of product amplification; the first cycle consisted of a denaturation step at 95 °C for 1 min, followed by annealing and elongation at 60 °C for 30 s and 72 °C for 1.5 min, respectively. All subsequent cycles were executed under the same conditions, with the exception of the last cycle, where the elongation step was extended to 7 min. We found a linear range (log luciferase/GAPDH *versus* log cycle number) between 10–17; as such we chose 11–13 cycles depending on the type of experiment.

The PCR products were analyzed and verified by electrophoresis on 1.15% agarose gels in a Tris borate/EDTA buffer system as described previously (37). All gel photos were subjected to a digital imaging system (see above) for semi-quantitative measurements, and results were expressed as a ratio of luciferase/GAPDH PCR fragments.

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay Experiments—Confluent HSF in 4-well cluster plates (3–5 × 10^6 cells/well) from control and treated cells were carefully scraped into 1.5 ml of ice-cold phosphate-buffered saline and pelleted by brief centrifugation. Nuclear extracts were prepared as described previously (37).

Double-stranded oligonucleotides containing consensus and mutant sequences were from Geneka Biotechnology Inc. (Montreal, Quebec, Canada) and were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide

kinase (Promega, Madison, WI). The sense sequences of the oligos tested are as follows: NF-κB p50, 5′-GCC ATG GGG GGA TCC CCG AAG TCC-3′; NF-κB p50 mut, 5′-GCC ATG G**GC CGA** TCC CCG AAG TCC-3′; ATF-2, 5′-GAT TCA ATG ACA TCA CGG CTG TG-3′; ATF-2 mut, 5′-GAT TAC A**GA ACA TAG** CGG CTG TG-3′. Binding buffer consisted of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl$_2$, 4% glycerol, and 2.5 μg of poly(dI-dC). Binding reactions were conducted with 15 μg of nuclear extract and 100,000 cpm of ^{32}P-labeled oligonucleotide probe at 22 °C for 20 min in a final volume of 10 μl. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis through 6% gels in a Tris borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography.

Eicosanoid Enzyme-linked Immunosorbent Assay (ELISA)—Measurements of the principal eicosanoids produced by HSF were performed by ELISA kits according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). Detection limits for PGE$_2$, LTB$_4$, and PGD$_2$ were 39, 3.9, and 7.8 pg/ml, respectively.

RESULTS

Kinetic Paradigm of rhIL-1β Induction of COX-2 Gene in HSF—Studies in our laboratory (39) indicate that the EC$_{50}$ for rhIL-1β-dependent *COX-2* gene induction in our cell culture system is 5.7 pmol/liter or 100 pg/ml. As such, this concentration was chosen for all subsequent experimentation. Time course studies revealed that increases in COX-2 mRNA expression were detectable within 5 min (1.4-fold), reached greater than 10-fold after 1 h, attained steady state at 3–4 h, and thereafter declined very gradually for the next 44 h as shown in Fig. 1, *A* and *B*. The COX-2 protein was perceptible within 1 h following stimulation with rhIL-1β and thereafter followed a pattern of biosynthesis closely resembling COX-2 mRNA expression (Fig. 1*C*). The rhIL-1β-stimulated PGE$_2$ release profile is also illustrated in Fig. 1*D* (mean, *n* = 4). The eicosanoid levels reached 21 ng per 10^6 cells after 1 h and 130 ng by 4 h, and by 24 h we detected more than 6000 ng per 10^6 cells. Other arachidonic acid metabolites elaborated by HSF, such as PGD$_2$ and LTB$_4$, were also released in higher concentrations after IL-1β-stimulation; however, in both cases, the amounts detected approximated 200 pg/ml per 10^6 cells (data not shown).

Suppression of rhIL-β-induced COX-2 Gene Expression by COX Inhibitors, Role of PGE$_2$—We reported (39) that the COX-2 preferential inhibitor NS-398 and its structural analog nimesulide inhibit rhIL-1β-induced COX-2 mRNA expression (maximum ~80%), COX-2 protein synthesis (maximum ~80%), and PGE$_2$ release (maximum ~95%) in a dose-dependent manner. The concentrations used represent sub- and therapeutic concentrations. In the present study, when PGE$_2$ was added to the incubation medium in increasing concentrations along with NS-398 and rhIL-1β, the inhibitory effect of NS-398 on COX-2 mRNA and protein synthesis was completely reversed (Fig. 2, *A* and *B*); PGE$_2$ didn't potentiate the inductive effect of rhIL-1β (Fig. 2*A*).

It is widely acknowledged that IL-1β and tumor necrosis factor-α (TNF-α) have overlapping proinflammatory effects, activate common signaling cascades, and induce similar target genes (reviewed in Ref. 42). As such we wondered whether TNF-α would also require PGE$_2$ to mediate the induction of COX-2. Previously, we showed that TNF-α, at physiologically relevant levels (*e.g.* 1–10 ng/ml or 60–60 pmol/liter), induces COX-2 mRNA expression, COX-2 protein synthesis, and PGE$_2$ release in HSF (43); here we demonstrate that the induction was not abrogated with the addition of NS-398, and in fact the inductive effect was potentiated with the addition of increasing concentrations of PGE$_2$ (Fig. 2*C*). In carefully controlled comparisons of IL-1β *versus* TNF-α stimulation of COX-2 expression and PGE$_2$ release in our cell culture system, we observed that IL-1β is at least 15-fold more potent in terms of COX-2 mRNA expression (ratio 19 to 1 COX-2 mRNA normalized to
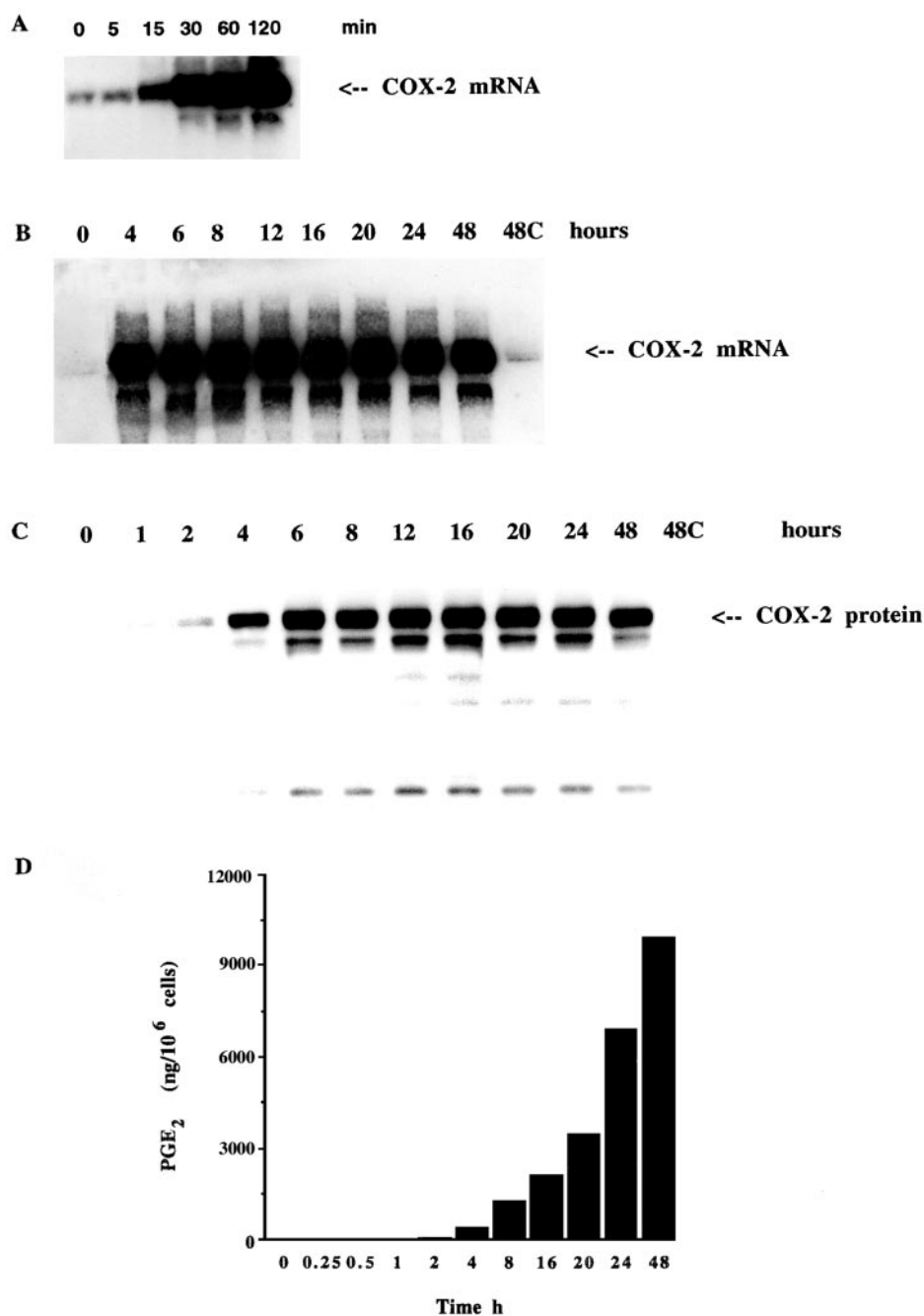
FIG. 1. **Time course of rhIL-1β stimulation of COX-2 mRNA (*A* and *B*), COX-2 protein (*C*), and PGE$_2$ release (*D*).** Cultured confluent human synovial fibroblasts (HSF, 1.2×10^6 cells in 6-well cluster plates) were preincubated for 24 h in DMEM supplemented with 1% FCS plus antibiotics at 37 °C in order to ensure synchrony and quiescence. Cells were then treated for varying times (0–48 h) with 5.7 pmol/liter (100 pg/ml) of rhIL-1β. Monolayers were extracted for protein or RNA; 50 μg of protein were analyzed for COX-2 protein by Western blotting using a specific polyclonal anti-COX-2 antiserum, whereas 5 μg of total RNA were analyzed for COX-2 mRNA by Northern hybridization using a specific DIG-labeled cDNA probe as described under "Experimental Procedures." Conditioned medium was collected and analyzed by ELISA for PGE$_2$. Results are expressed as the mean of 4 separate experiments performed in duplicate. Interassay coefficient of variation never exceeded 13% when expressed as ng/10^6 cells.

GAPDH, $n = 3$) and more than 40 times more active with respect to PGE$_2$ release (6.5 μg of PGE$_2$/10^6 cells/24 h *versus* 0.159 μg of PGE$_2$/10^6 cells/24 h, $n = 3$).

Interestingly, the inflammatory 5-lipoxygenase product LTB$_4$ could also reverse NS-398-dependent inhibition of IL-1β-induced COX-2 mRNA expression and protein synthesis, whereas a putative anti-inflammatory prostanoid and PPAR agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$, had no effect (Fig. 3). All three arachidonic acid metabolites had absolutely no effect on the *COX-2* gene in quiescent, unstimulated cells. The nonselective COX-2 inhibitor indomethacin (0.5–5 μg/ml) also inhibited rhIL-1β-induced COX-2 mRNA expression (maximum $\sim$80%) and COX-2 protein synthesis (maximum $\sim$80%) but in an inverse, dose-dependent manner (Fig. 3). As expected, PGE$_2$ release was blocked by at least 95% at 5 μg/ml (data not shown).

p38 MAP Kinase Activity and PGE$_2$-dependent Regulation of COX-2 Gene Expression in IL-1β-treated Cells—Previous stud-

ies in our laboratory demonstrate that calcium channel blockers (*e.g.* verapamil and nifedipine) and reactive oxygen species scavengers (*e.g.* pyrrolidinedithiocarbamate, *N*-acetyl-L-cysteine, and L-*N*^{6}-(1-iminoethyl)lysine) exert either no effect or very modest inhibition on rhIL-1β-stimulated COX-2 mRNA and protein synthesis (39). In this study, SB202190 (SAPK/p38α inhibitor) suppressed rhIL-1β-induced COX-2 mRNA and protein synthesis by $\sim$80% (78 $\pm$ 9%, mean $\pm$ S.D., $n = 7$) (Fig. 4, *A* and *B*); PGE$_2$ release was suppressed by greater than 95% (data not shown). The MEK1/2 inhibitor PD98059, and the IκB kinase inhibitor, Bay-11-7802 were without significant effect, whereas the PKA inhibitor KT-5720 had modest but consistent inhibitory activity. In order to determine whether the suppressive effect of SB202190 was also dependent on PGE$_2$ release (as in Fig. 2, *A* and *B*), we co-incubated 10–1000 nmol/liter of PGE$_2$ with SB202190 and rhIL-1β and found that the eicosanoid restored about 20–25% (23.08 $\pm$ 2.92%, mean $\pm$ S.D., $n = 7$) of the levels observed with rhIL-1β alone (Fig. 4C). As such, these

PGE₂ Regulates COX-2 mRNA Stability and Translation

A

B

C
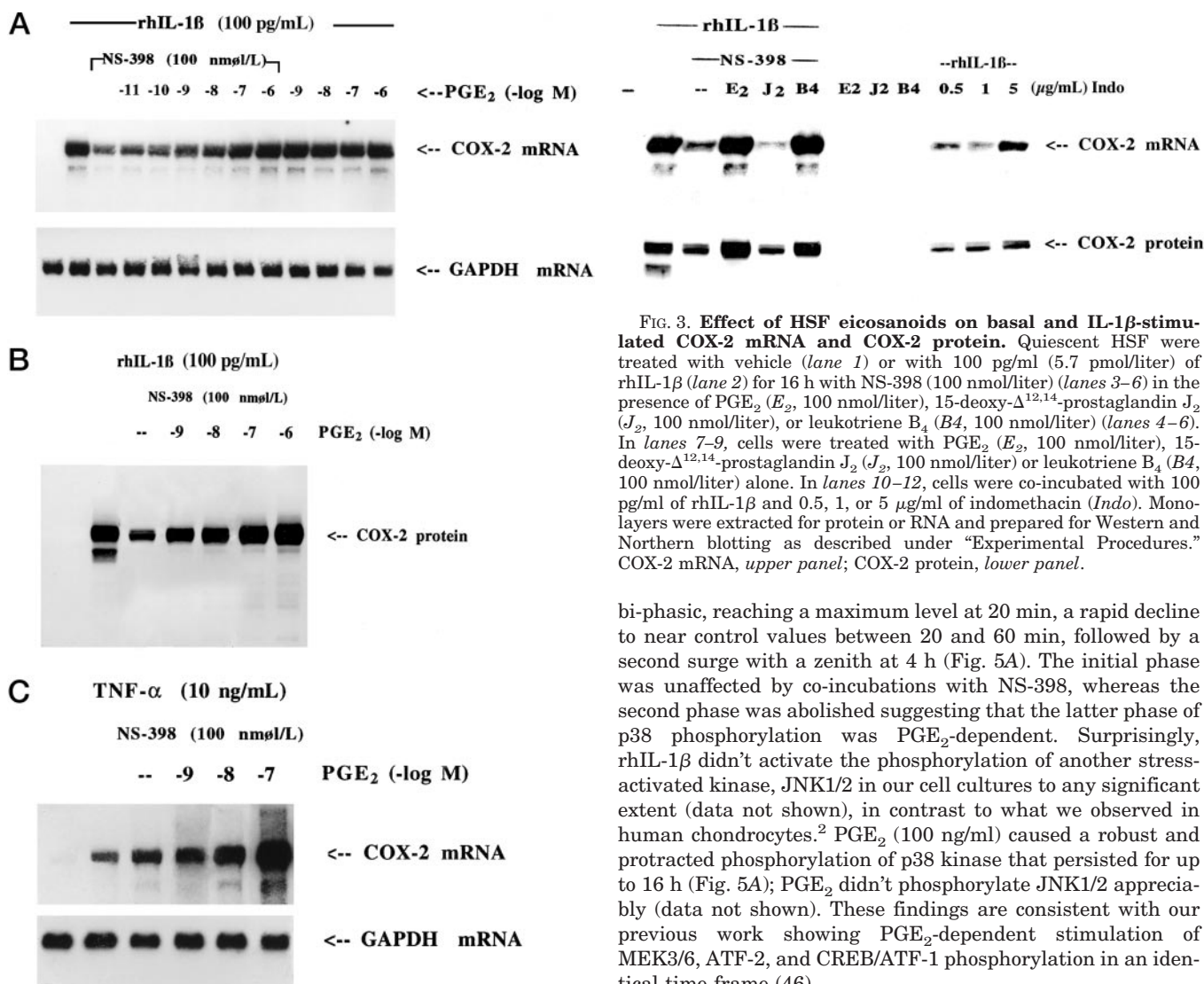
FIG. 2. **rhIL-1β stimulation of COX-2 mRNA (*A*) and COX-2 protein (*B*) is PGE₂-dependent.** TNF-α stimulation of COX-2 mRNA is PGE₂-independent (*C*). Quiescent HSF were treated with 100 pg/ml (5.7 pmol/liter) of rhIL-1β (*A* and *B*) or 10 ng/ml of TNF-α (*C*) for 16 h with or without NS-398 (100 nmol/liter) in the absence or presence of increasing concentrations of PGE₂ (10 pmol/liter to 1 μmol/liter, −*log M*, −11 to −6, as per figure). Monolayers were extracted for protein or RNA and prepared for Western and Northern blotting as described under "Experimental Procedures." Blots are representative of three separate experiments that gave essentially identical results.

results suggested that part of PGE₂-dependent effect (compare Fig. 2, *A* and *B* with Fig. 4, *A–C*) was not affected by the presence of SB202190 (*i.e.* not p38 MAP kinase-dependent). As such we investigated the cAMP/PKA signaling cascade, which is also potently stimulated by PGE₂ via the prostaglandin EP4 receptor in our cell cultures (44, 45). We co-incubated the cAMP mimetics rolipram, a cAMP-dependent phosphodiesterase type IV inhibitor, and forskolin, an activator of adenylate cyclase, with SB202190 in order to examine the role of the cAMP signaling cascade of COX-2 mRNA stability. The levels of COX-2 mRNA were reactive to elevations in cellular cAMP and were restored to about 15% (15.05 ± 1.83%, mean ± S.D., n = 7) of the rhIL-1β-induced levels in the face of p38 MAP kinase blockade (Fig. 4*D*).

The activation by pro-inflammatory cytokines of the p38 kinase cascade is well described, although the role of prostanoids in this regard is not clear (reviewed in Ref. 28). In HSF, the stimulation of p38 kinase phosphorylation by rhIL-1β was
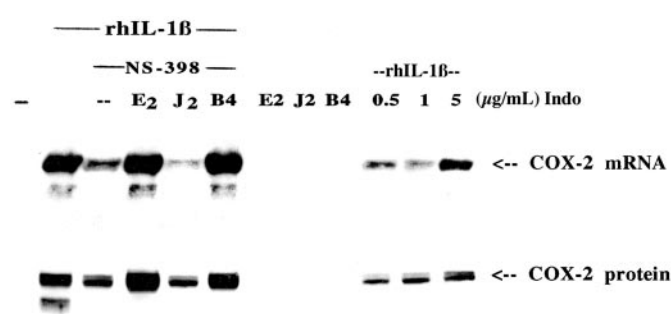
FIG. 3. **Effect of HSF eicosanoids on basal and IL-1β-stimulated COX-2 mRNA and COX-2 protein.** Quiescent HSF were treated with vehicle (*lane 1*) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β (*lane 2*) for 16 h with NS-398 (100 nmol/liter) (*lanes 3–6*) in the presence of PGE₂ (*E₂*, 100 nmol/liter), 15-deoxy-Δ¹²,¹⁴-prostaglandin J₂ (*J₂*, 100 nmol/liter), or leukotriene B₄ (*B4*, 100 nmol/liter) (*lanes 4–6*). In *lanes 7–9*, cells were treated with PGE₂ (*E₂*, 100 nmol/liter), 15-deoxy-Δ¹²,¹⁴-prostaglandin J₂ (*J₂*, 100 nmol/liter) or leukotriene B₄ (*B4*, 100 nmol/liter) alone. In *lanes 10–12*, cells were co-incubated with 100 pg/ml of rhIL-1β and 0.5, 1, or 5 μg/ml of indomethacin (*Indo*). Monolayers were extracted for protein or RNA and prepared for Western and Northern blotting as described under "Experimental Procedures." COX-2 mRNA, *upper panel*; COX-2 protein, *lower panel*.

bi-phasic, reaching a maximum level at 20 min, a rapid decline to near control values between 20 and 60 min, followed by a second surge with a zenith at 4 h (Fig. 5*A*). The initial phase was unaffected by co-incubations with NS-398, whereas the second phase was abolished suggesting that the latter phase of p38 phosphorylation was PGE₂-dependent. Surprisingly, rhIL-1β didn't activate the phosphorylation of another stress-activated kinase, JNK1/2 in our cell cultures to any significant extent (data not shown), in contrast to what we observed in human chondrocytes.[2] PGE₂ (100 ng/ml) caused a robust and protracted phosphorylation of p38 kinase that persisted for up to 16 h (Fig. 5*A*); PGE₂ didn't phosphorylate JNK1/2 appreciably (data not shown). These findings are consistent with our previous work showing PGE₂-dependent stimulation of MEK3/6, ATF-2, and CREB/ATF-1 phosphorylation in an identical time frame (46).

Gel-shift analysis revealed that SB202190 (500 nmol/liter to 10 μmol/liter) inhibited rhIL-1β-induced ATF-2 oligonucleotide binding but caused a mild induction of NF-κB (p50) nuclear binding proteins both in the absence and presence of IL-1β (data not shown).

In order to examine for elements of transcriptional control of COX-2 via p38 MAP kinase or cAMP/PKA signaling, we conducted transient transfection analyses and observed that rhIL-1β (100 pg/ml) stimulated a human COX-2 promoter-luciferase reporter construct by 1.99 ± 0.14-fold (mean ± S.D., n = 4); this induction was not inhibited by NS-398 nor PGE₂ but was completely abrogated by SB202190 (Fig. 5*B*). Neither NS-398, PGE₂, nor SB202190 had any effect on basal promoter activity. In companion experiments, co-expression of a MEK3 expression vector stimulated the COX-2 promoter luciferase construct by 1.76 ± 0.23-fold (mean ± S.D., n = 4); SB202190 abolished the induction completely (Fig. 5*B*). The magnitude of COX-2 promoter activation by rhIL-1β and MEK3 was not significantly different. The overexpression of catalytic subunits of PKA had no effect on the COX-2 promoter activity (Fig. 5*B*), whereas co-expression of MEK3 and PKA_cat synergized to increase COX-2 promoter activity to 3.26 ± 0.33-fold (mean ± S.D., n = 4), an effect partially inhibited by SB202190 (Fig. 5*B*).

Prostaglandin E₂ Stabilizes COX-2 mRNA—Judging by the

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[2] W. H. Faour, Y. He, Q. W. He, M. de Ladurantaye, M. Quintero, A. Mancini, and J. A. Di Battista, unpublished observations.
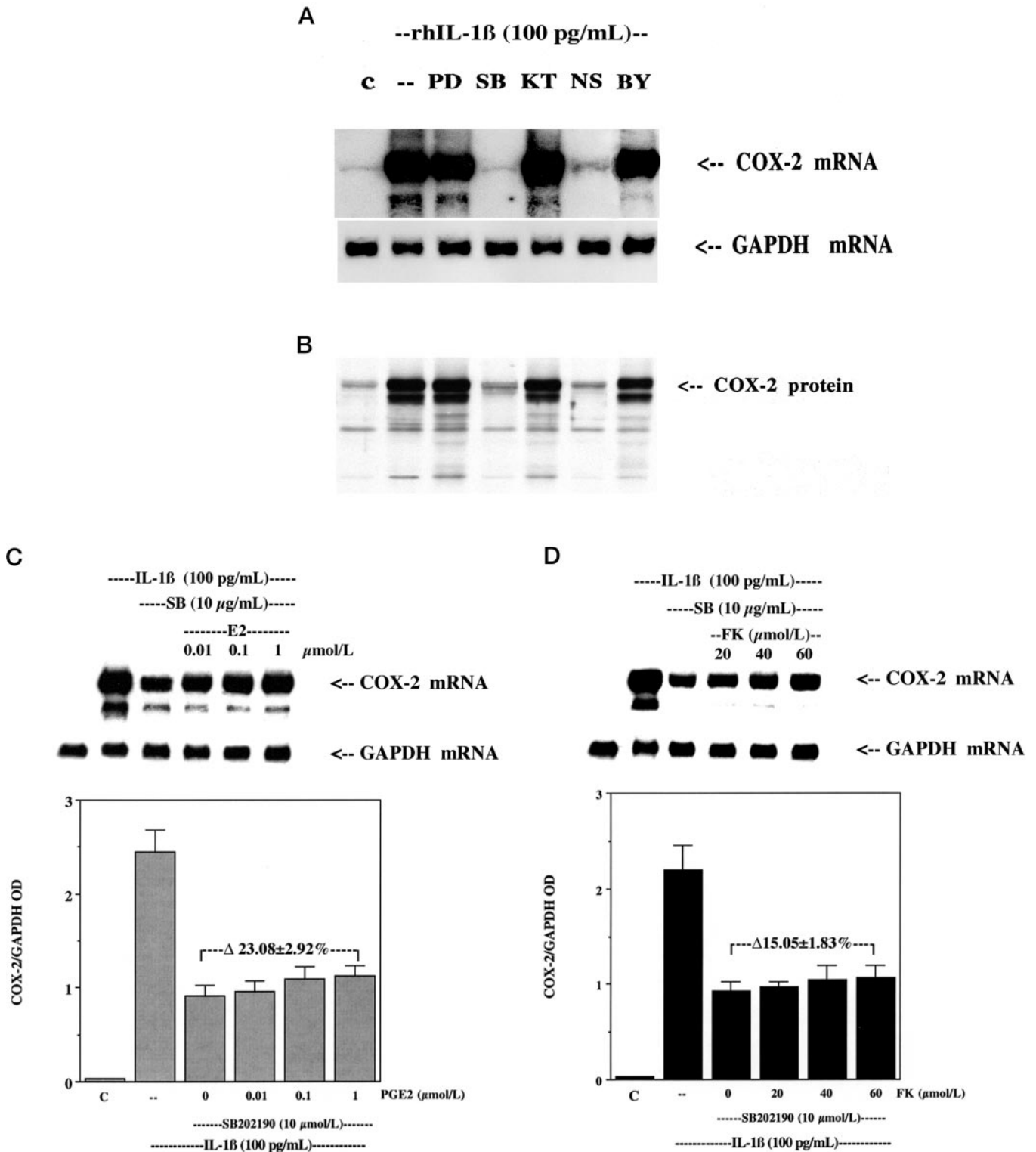
FIG. 4. **Effect of protein kinase inhibitors on rhIL-1β stimulation of COX-2 mRNA (*A*) and COX-2 protein (*B*).** Possible mediation by p38 MAP kinase and protein kinase A (*C* and *D*). Quiescent HSF were treated with vehicle (*c, lane 1*) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β (*lane 2*) for 16 h in the presence of either PD98059 (*PD,* 50 μmol/liter), a MEK1/2 inhibitor, SB202190 (*SB,* 10 μmol/liter), a p38 MAP kinase inhibitor, or KT-5720 (*KT,* 2 μmol/liter), a PKA inhibitor, NS-398 (*NS,* 100 nmol/liter), or Bay-11–7082 (*BY,* 5 μmol/liter), an IKK inhibitor. Monolayers were extracted for protein or RNA; 5 μg of total RNA were analyzed for COX-2 mRNA and GAPDH mRNA by Northern hybridization using specific DIG-labeled cDNA probes, whereas 50 μg of protein were analyzed for COX-2 protein by Western blotting using a specific polyclonal anti-COX-2 antiserum as described under "Experimental Procedures." *C* and *D*, cells were incubated with rhIL-1β in presence of SB202190 in the absence or presence of increasing concentrations of PGE$_2$ (*E$_2$*, 0.01–1 μmol/liter) or the cAMP mimetics forskolin (20–60 μmol/liter) + rolipram (100 μmol/liter) as per the figure. Northern analysis was performed as described above. *C,* *$p < 0.01$; *D,* **$p < 0.04$, Student's *t* test.

accumulated data (see above), it is unlikely that PGE$_2$ modifies COX-2 expression in HSF at the transcriptional level. As such we examined post-transcriptional mechanisms involving

strictly message stabilization and/or protein translation. As a first approach we employed classical techniques involving measuring COX-2 mRNA in transcriptionally arrested cells

PGE₂ Regulates COX-2 mRNA Stability and Translation

FIG. 5. *A,* time course of p38 MAP kinase phosphorylation (Thr-80/Tyr-82) by rhIL-1β and PGE₂. Quiescent HSF treated for varying times (0–16 h, *16C* = 16 h control) with 100 pg/ml (5.7 pmol/liter) of rhIL-1β (*top panel*), rhIL-1β + NS-398 (100 nmol/liter) (*middle panel*), or PGE₂ (100 nmol/liter) (*lower panel*). Monolayers were extracted for protein, and 50 μg were analyzed for total and phospho-p38 MAP kinase by Western blotting using a specific rabbit polyclonal antisera as described under "Experimental Procedures." *B,* transcriptional activation of the COX-2 promoter by IL-1β and the role of p38 MAP kinase. Cells were plated at 40% confluence in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Two μg of the COX-2 promoter-luciferase construct, in the presence or absence of 50 ng each of pFCMEK3, pCMV, or pFCPKA$_{cat}$, or pFCMEK3 + pFCPKA$_{cat}$ and 0.5 μg of pCMV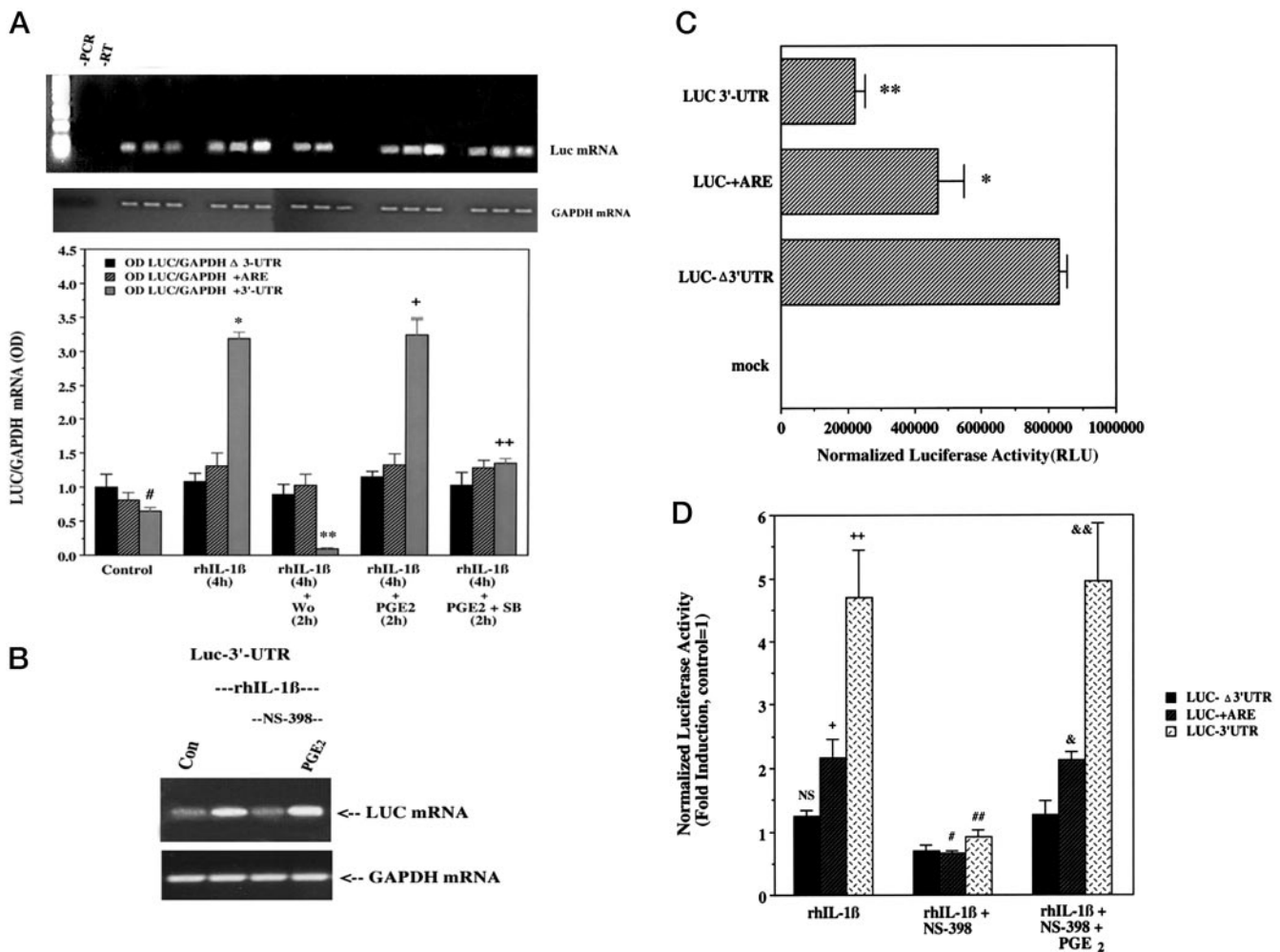-β-galactosidase were transfected for 6 h using LipofectAMINE 2000 according to the manufacturer's instructions. Following a change in medium containing 1% FCS (2 h), transfected cells were incubated for 6 h with biological effectors rhIL-1β (5.7 pmol/liter), NS-398 (100 nmol/liter), SB202190 (10 μmol/liter), and PGE₂ (100 nmol/liter) as per the figure. Cells were lysed, and the luciferase activity, β-galactosidase activity, and protein content were determined as described under "Experimental Procedures." *, $p < 0.005$ *versus* control; +, $p < 0.01$ *versus* IL-1β; ++, $p < 0.01$ *versus* pFCMEK3; $p < 0.05$ *versus* pFCMEK3 + pFCPKA$_{cat}$ (mean ± S.D., $n = 8$ determinations in duplicate).

(actinomycin D) in the absence or presence of PGE₂. When HSF were activated with rhIL-1β for 3–4 h (steady state) followed by washout and a fresh change of medium, the elevated levels of COX-2 mRNA declined rapidly ($t_{1/2}$ = 0.85 h, n = 4 determinations) such that within 2 h the levels were similar to control, unstimulated cells (Fig. 6, *A* and *B*). The inclusion/exclusion of actinomycin D (1 μg/ml) had no effect on the rate of COX-2 mRNA decay in these experiments (data not shown). However, if PGE₂ (1 μg/ml) was added to fresh medium (in the presence of actinomycin D) COX-2 mRNA levels remained elevated for up to 16 h ($t_{1/2}$ = 13.02 h, n = 4 determinations) and declined thereafter (Fig. 6, *A* and *B*). The addition of a monoclonal anti-PGE₂ antibody, but not a monoclonal anti-LTB₄ antibody (control), abolished the stabilizing effect suggesting the exogenously added PGE₂ alone was responsible for stabilizing COX-2 mRNA. Although we showed that long term phospho-

rylation of p38 kinase by IL-1β was PGE₂-dependent (see Fig. 5*A*), it was necessary to establish whether COX-2 mRNA stabilization by PGE₂ was also a p38 kinase-mediated process. Indeed, SB202190 reduced considerably the stabilization of COX-2 mRNA by PGE₂ (Fig. 6, *C* and *D*), whereas the PKA inhibitor KT-5720 was somewhat effective in this regard ($14.21 \pm 3.39\%$, n = 4).

As we have shown (45), HSF express trace quantities of EP3 receptors (isotype I) that mediate either calcium fluxes or decreases in cellular levels of cAMP. The predominant EP4 receptor is associated with increases in cellular cAMP levels (44–46) and is coupled to MAP kinase cascades by Rap1/B-Raf activation (reviewed in Ref. 47). Butaprost (EP2 > P3 agonist) and sulprostone (EP1 > EP3 agonist) (data not shown) had little stabilizing effect on COX-2 mRNA up to 10 μmol/liter (Fig. 6*E*, compare PGE₂ *versus* butaprost).

As mentioned earlier, the COX-2 mRNA has multiple copies of the Shaw-Kamen AU-rich sequences that are believed to influence message stability. Recent studies (36, 41) have provided evidence that the AU-rich elements (6) in the first 116 bp of the 3′-UTR may mediate COX-2 mRNA instability. Furthermore, specific cytoplasmic AU-binding proteins were identified that may initiate message degradation (36, 41). To determine whether PGE_2/p38 MAP kinase-dependent mRNA stabilization is manifested through the 3′-UTR and AU-rich sequences, we transfected HSF with cytomegalovirus-driven chimeric expression constructs containing luciferase cDNA (reporter) fused to the COX-2- 3′-UTR (Luc + 3′-UTR), AU-rich region (Luc + ARE), or complete removal of the 3′-UTR region (Luc Δ3′UTR) (kindly provided by Dr Stephen Prescott, University of Utah). By using an identical experimental design as in Fig. 6*A*, we observed that rhIL-1β stimulated luciferase mRNA after 4 h stimulation by more than 3-fold using Luc + 3′-UTR constructs (Fig. 7*A, top*, representative RT-PCR; *lower panel*, densitometric analysis of five determinations). Removal of the rhIL-1β stimulus for 2 h under washout conditions resulted in a substantial (apparently rapid) decrease in luciferase mRNA. The addition of PGE_2 reversed the dramatic fall in luciferase mRNA levels, an effect largely reversed by the co-addition of the p38 kinase inhibitor SB202190. As expected, HSF transfected with constructs devoid of COX-2 3′-UTR were unresponsive to our treatment protocols, whereas the inclusion of the AU-rich region of COX-2 3′-UTR resulted in more modest effects on luciferase mRNA expression. No major mRNA decay was observed in the latter experiments for an additional 4 h, whereas the presence of the entire 3′-UTR region resulted in complete disappearance of Luc mRNA under our experimental conditions (data not shown). In addition, when we treated cells transfected with the Luc + 3′-UTR construct followed by treatments with IL-1β in the absence or presence of NS-398, with or without PGE_2, rhIL-1β substantially augmented luciferase mRNA after 16 h of stimulation. The stimulation was abrogated by the addition of NS-398 to a substantial degree; the latter inhibitory effect was entirely reversed by further additions of PGE_2 (Fig. 7*B*).

PGE_2 Mitigates the Inhibition of Protein Translation by 3′-UTR Region of COX-2 mRNA—There is now good evidence that the AU-rich elements of certain cytokines and COX-2 mRNA inhibit translation (41, 48–50). By using the same chimeric constructs transiently transfected in our primary cultures of HSF as described above, we found dramatically increased luciferase activity (protein) when the 3′-UTR was removed (Luc Δ3′UTR COX-2) as compared with Luc + 3′-UTR or Luc + ARE (Fig. 7*C*). The entire 3′-UTR COX-2 region mediated the most robust inhibition of translation with the AU-rich region (proximal 116 bp) displaying intermediate inhibitory activity in this regard (Fig. 7*C*). In order to assess whether translational mechanisms are implicated in the induction of COX-2 protein

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FIG. 6. **PGE_2-dependent stabilization of COX-2 mRNA is mediated by p38 MAP kinase and protein kinase A.** Quiescent HSFs were treated with vehicle (*c*) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β (*I*) for 4 h (steady state) after which time cells were washed out (*wo*) and treated with actinomycin D (1 μg/ml) for 30 min, and then fresh medium was added containing either vehicle (*wo*) or PGE_2 (*E₂*, 100 nmol/liter). After an additional 2, 4, 8, or 16 h of incubation, monolayers were extracted for RNA at each time point, and 5 μg of total RNA were analyzed for COX-2 mRNA and GAPDH mRNA by Northern hybridization using specific DIG-labeled cDNA probes (*A*). *B,* densitometric analysis of COX-2 mRNA decay in the presence or absence of PGE_2 (*n* = 4 determinations). *C–E,* cells were treated with vehicle (*c*) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β (*I*) for 4 h, washed out (*wo*), treated with actinomycin D (1 μg/ml) for 30 min, and then fresh medium was added containing either vehicle (*wo*) or PGE_2 (*E₂*, 100 nmol/liter) alone or in the presence of increasing amounts of a monoclonal anti-PGE_2 antibody or, as a control, a monoclonal anti-LTB_4 antibody (*C*), SB202190 (*SB*, 10 μmol/liter) or KT-5720 (*KT*, 2 μmol/liter) (*D*), or increasing concentrations of PGE_2 (*E₂*, 0.01–1 μmol/liter) or butaprost (*BU*, 0.1–10 μmol/liter) (*E*) for 4 more hours.

FIG. 7. **PGE₂-dependent stabilization of COX-2 mRNA and increased translation is mediated by the proximal (AU-rich) and distal portion of COX-2 3′-UTR.** Cells were plated at 40% confluence in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Two μg of LUC-3′-UTR, LUC-+ARE, or LUC-Δ3′-UTR and 0.5 μg of pCMV-β-galactosidase were transfected for 6 h using FuGene 6™ according to the manufacturer's instructions. Following a change in medium containing 1% FCS (2 h), transfected cells were treated with vehicle (control) or with 100 pg/ml (5.7 pmol/liter) of rhIL-1β for 4 h, washed out (*wo*), treated with actinomycin D (1 μg/ml) for 30 min, and then fresh medium was added containing either vehicle (*wo*) or PGE₂ (*E₂*, 100 nmol/liter) alone or in the presence of SB202190 (*SB*, 10 μmol/liter) for an additional 2 h (*A*). Monolayers were scraped into phosphate-buffered saline, divided into 2 portions, centrifuged, and extracted for protein to determine β-galactosidase activity or extracted for RNA, and 1 μg of total RNA was analyzed for luciferase mRNA and GAPDH mRNA by RT-PCR as described under "Experimental Procedures." A representative RT-PCR gel is shown along with statistical analysis of densitometric data (*n* = 5). #, *p* < 0.02 *versus* control LUC-Δ3′-UTR; *, *p* < 0.0005 *versus* control LUC-3′-UTR; **, *p* < 0.0001 *versus* rhIL-1β LUC-3′-UTR; +, *p* < 0.0002 *versus* rhIL-1β + *Wo* (2 h) LUC-3′-UTR; ++, *p* < 0.001 *versus* rhIL-1β + PGE₂ (2 h) LUC-3′-UTR. *B*, cells were incubated for 16 h with rhIL-1β (100 pg/ml), rhIL-1β + NS-398 (100 nmol/liter), or rhIL-1β + NS-398+ PGE₂ (100 nmol/liter). Monolayers were extracted for RNA, and 1 μg of total RNA was analyzed for luciferase mRNA and GAPDH mRNA by RT-PCR. *C* and *D*, 2 μg of LUC-3′-UTR, LUC-+ARE, or LUC-Δ3′-UTR and 0.5 μg of pCMV-β-galactosidase were transfected for 6 h using FuGene 6™ followed by a change in medium containing 1% FCS (2 h). Cells were then analyzed for luciferase activity (*C*) or incubated for 16 h with biological effectors rhIL-1β (100 pg/ml), rhIL-1β + NS-398 (100 nmol/liter), or rhIL-1β + NS-398+ PGE₂ (100 nmol/liter) as per the figure (*D*). Cells were lysed and the luciferase activity, β-galactosidase activity, and protein content were determined as described under "Experimental Procedures." *n* = 3 determinations; *, *p* < 0.01 *versus* LUC-Δ3′-UTR; **, *p* < 0.001 *versus* LUC-Δ3′-UTR; +, *p* < 0.03 *versus* control; ++, *p* < 0.001 *versus* control; #, *p* < 0.01 *versus* rhIL-1β; ##, *p* < 0.001 *versus* rhIL-1β; &, *p* < 0.001 *versus* rhIL-1β + NS-398; &&, *p* < 0.001 *versus* rhIL-1β + NS-398.

synthesis by IL-1β and whether the effect may be PGE₂-dependent, we transfected HSF with the chimeric constructs and treated the cells with IL-1β in the absence or presence of NS-398 with or without PGE₂. Indeed rhIL-1β substantially increased luciferase activity (protein synthesis) after 16 h of stimulation, and the effect proved to be almost entirely PGE₂-dependent (compare data, *rhIL-1β+NS-398 versus rhIL-1β+NS-398+PGE₂,* Fig. 7*D*). Interestingly, the AU-rich region of COX-2 mRNA appeared to play a modest role in the PGE₂-dependent increase in protein synthesis with the distal AUUUA sequences being apparently more important in this regard (Fig. 7*D*).

DISCUSSION

Normally not expressed, the elevated levels of COX-2 mRNA, COX-2 protein, and PGE₂ release observed in OA/RA-affected synovial membranes have been associated etiologically with the disease process (17, 18). Thus understanding the mechanisms responsible for aberrant COX-2 expression is of considerable clinical concern. It now seems clear from published evidence and the present study that the *COX-2* gene is regulated through both 5′ (transcriptional) and 3′ (post-transcriptional) regulatory elements following IL-1β signal activation in both normal (this study) and transformed cell phenotypes. We believe that the levels of IL-1β-induced COX-2 mRNA are the

result of a more minor element of transcriptional control supported by robust post-transcriptional regulation (*i.e.* message stability and/or translational control). The COX-2 protein levels always mirrored precisely COX-2 mRNA in our studies, suggesting precise coordination between message expression and translation. Our data confirm previous work (14, 35, 36) implicating the p38 MAPK cascade as critical to the magnitude and duration of cellular COX-2 mRNA. We now add, however, that the COX-2 product, PGE$_2$, is largely responsible for this activation of p38 MAPK cascade resulting in the stabilization of COX-2 mRNA and increased COX-2 protein synthesis. In this regard, the exact contribution of the cAMP/PKA pathway (see "Results") will require further investigation but is likely to be another example of signaling redundancy (51). In this connection, our COX-2 promoter experiments seem to suggest that p38 MAP kinase and the cAMP/PKA synergize to activate transcription so that expression of the *COX-2* gene may be controlled transcriptionally and post-transcriptionally by the latter two signaling pathways. The data are also consistent with a positive feedback loop involving released PGE$_2$ binding to the prostaglandin EP4 receptor in our cell cultures. Therefore, considering the impressive amounts of PGE$_2$ released by human OA/RA synovial membranes (in explant cultures (52)), it is conceivable that the *COX-2* gene expression levels are sustained in this cyclic, positive-feedback loop.

There are contrary reports (53, 54) of PGE$_2$ inhibiting IL-1β-induced COX-2 expression in human umbilical vein endothelial cells presumably through a negative feedback loop. The design of the latter studies was different to the extent that high concentrations of exogenous PGE$_2$ were added in co-incubation with IL-1β and, in some cases, in the absence of any inhibition of endogenous prostanoid production (*e.g.* by NS-398). One explanation could be that added PGE$_2$ inhibits IL-1β-induced NF-κB-driven transactivation of the COX-2 promoter by interacting with IκB kinase, although this may require metabolism of PGE$_2$ to cyclopentenone derivatives by human umbilical vein endothelial cells (55). It is curious that IL-1β-induced PGE$_2$ release occurs in tandem with increases with COX-2 mRNA and protein, whereas exogenously added PGE$_2$ reverses the up-regulation. Compared with HSF, human umbilical vein endothelial cells release much less PGE$_2$ and thus COX-2 expression could not depend on PGE$_2$ accumulation (54). The phenomenon may be particular to endothelial cells since we have observed only PGE$_2$-dependent positive feedback in human macrophages, chondrocytes, and skin fibroblasts.[2]

The present data are consistent with a more minor element of transcriptional regulation of the *COX-2* gene by IL-1β through the p38 kinase pathway occurring within the 1st hour or so. By using nuclear run-on assays, however, others have shown (56, 57) 5–15-fold increases in COX-2 transcriptional rates following IL-1β stimulation which would better reflect steady-state mRNA levels normally observed. We were able to measure only a 2-fold increase in human COX-2 promoter (-1840 bp) activity after IL-1β stimulation of transiently transfected HSF; addition of NS-398, PGE$_2$, or 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ had no effect, although SB202190 was capable of reversing promoter induction. Furthermore, MEK3 overexpression induced human COX-2 promoter activity to levels similar those induced by rhIL-1β, and this was also quite sensitive to SB202190. The p38 MAP kinase pathway synergized with the cAMP/PKA cascade to elicit transcriptional activation. Saklatvala and co-workers (58) observed, using nuclear run-on technology and HeLa cells, that IL-1β induced a 2-fold increase in transcription of the *COX-2* gene and that the effect was refractory to inhibitors of p38 kinase. Since PGE$_2$ incubated alone doesn't stimulate transcription of the *COX-2*

gene, the massive increase and persistence of COX-2 mRNA is most likely due, in large part, to post-transcriptional regulation. Nevertheless, even considering the strong evidence for COX-2 mRNA stabilization, one must include some transcriptional control since both NS-398 and SB202190 inhibit 80–90%, not 100%, of IL-1β-induced COX-2 mRNA. Although PGE$_2$ stabilized COX-2 mRNA for up to 16 h in transcriptionally arrested cells, 24 h COX-2 mRNA levels in the presence of PGE$_2$ were no different from controls. We attribute this to rapid PGE$_2$ metabolism (59, 60), an effect that would not be as critical with cells constantly releasing PGE$_2$ as in the presence of IL-1β for 0–48 h (see Fig. 1).

There is some circumstantial evidence suggesting that NF-κB mediates a transcriptional induction of the *COX-2* gene by IL-1 in human RA synovial fibroblasts (25). These latter studies, however, were not designed to address the mechanisms controlling steady-state COX-2 mRNA levels and were focused on the notion of NF-κB-dependent promoter-based regulation. However, no data were reported to show a direct activation of the COX-2 reporter by NF-κB via its cognate response element(s). Evidence of this nature has been demonstrated in osteoblasts and vascular endothelial cells, as mentioned earlier (26, 27). Nevertheless, it could be argued that the NF-κB cascade acts as a "priming" mechanism in our cell cultures to initiate transcription of the gene, while the PGE$_2$-dependent p38 kinase signaling pathway stabilizes, augments, and maintains the levels of COX-2 mRNA found in cells after an IL-1β signal. The results with TNF-α may be instructive in this regard. Consider that the TNF-α-induced response, in terms of COX-2 mRNA/protein and PGE$_2$ release, amounts to about 2.5–5% of the cellular response normally induced by IL-1β[2]; it is not suppressed by co-incubations in the presence of NS-398, and further additions of PGE$_2$ potentiate the effect to the extent that the steady-state levels of COX-2 mRNA become indistinguishable from those generated by IL-1β. In HSF, TNF-α is an extremely potent activator of the NF-κB cascade (25, 43) while having little or no discernible effect on p38 kinase. Redress to chemical blockade of IKK yielded equivocal results and suppressed only a fraction of IL-1β-induced COX-2 mRNA expression; the latter portion was not PGE$_2$-reversible.[2] We chose to interpret these experiments with due caution as Bay-11-7082 (IKK inhibitor) at high concentrations (2-fold EC$_{50}$) is cytotoxic as are other NF-κB inhibitors especially those that block proteosomal activity. This observation is perhaps not surprising given that inhibition of IKK in primary and transformed cell lines causes apoptosis (61, 62). Using stable transfectants of HSF harboring either a wild type IκBα expression construct or a non-phosphorylatable IκBα mutant expression construct has not helped to resolve the transcriptional issue since there was no blockade of induced COX-2 expression by IL-1β or TNF-α,[2] suggesting purely post-transcriptional p38-dependent mechanisms. In a recent publication (43), we showed that the T-helper cell (Th2)-derived cytokines IL-4 and IL-10 inhibit, virtually to 100%, TNF-α/IL-1β-stimulated cPLA$_2$ protein, COX-2 mRNA, COX-2 protein; and PGE$_2$ release; tandem experiments reveal that the cytokines have no effect on TNF-α/IL-1β-induced NF-κB as judged by gel-shift analysis. However, in the same study, TNF-α/IL-1β-induced c/EBP activation was potently reversed by IL-4; the critical role of c/EBP in COX-2 promoter activation has been described previously (26, 27).

Recently it was shown (33) that the strength and duration of COX-2 mRNA expression following IL-1β activation of HeLa cells could be traced to the posttranscriptional regulatory phase in which a short (123-nucleotide) fragment of the COX-2 3'-UTR was shown to be essential and sufficient for the regu-

lation of mRNA stability by a p38/MAPKAPK-2/hsp 27 cascade. The latter nucleotide sequence interacted with a protein identified as an AU-rich-element/poly(U) binding factor I (36). The authors used posttranscriptional reporter constructs in which 3′-UTR fragments of COX-2 were cloned into a rabbit β-globin reporter plasmid under the control of a tetracycline operator sequence. Dominant-negative mutants of MAP-KAPK-2 and SB203580, a p38 inhibitor with properties similar to SB202190, blocked the MKK3/6 expression vector stabilization of the β-globin mRNA. Others (41, 63, 64) have used transient or stable transfections in immortalized cell lines using luciferase reporters with COX-2 3′-UTR fragments cloned downstream. Both stable and transient approaches came to the same conclusions about the importance of the AU-rich sequences in the 3′-UTR as targets for cytoplasmic/microsomal binding proteins that act to destabilize mRNA. However, the notion that mRNA stability was coupled to translational efficacy was presented, and it was argued that in fact AU sequence arrays regulate translation through a complex, yet-to-be-described interaction of AU-binding proteins and translational initiation factors. This notion is on solid ground based on data with AU-rich sequences from cytokine mRNAs (48, 49).

Our work suggests that the COX-2 3′-UTR inhibits protein (reporter) translation and promotes rapid mRNA decay in HSF. The proximal AU-rich region and distal AUUUA sequences contribute more or less equally to the translation inhibitory effect, although the entire 3′-UTR was seemingly far more important to mRNA decay than the AU-rich region alone. Regardless, the inhibition of translation and mRNA decay were relieved when the cells are treated with IL-1β; this was a purely PGE₂/p38 MAP kinase-dependent effect that reflects quite closely what we observed in our COX-2 mRNA experiments (Fig. 6*A*). In contrast to the previous studies emphasizing AU-rich tract-dependent regulation (33, 36, 41), we find that distal AUUUA sequences were quite reactive to the stabilizing effects of PGE₂ both in terms of mRNA and protein in HSF. Further studies will reveal whether the eicosanoid stimulates proteins that favor COX-mRNA stability (36, 50) and translation or inhibit those that serve to destabilize the message/translation. Whatever the case, it would not be surprising if we find that these proteins differ in quantity and/or composition from those isolated and identified from primate cells in heterologous systems (41) or transformed human cell lines (*e.g.* HeLa, 36).

We thus used cell-permeable pharmacological inhibitors like SB202190 and NS-398 to study COX-2 mRNA stability which always raise concerns about specificity and thus the validity of the data presented here. For example, SB202190 at higher concentrations can inhibit certain isoforms of JNK (65, 66), which could lead to erroneous conclusions about the predominant role of p38 MAP kinase. This proved not to be of concern for us since JNK is not phosphorylated in HSF following rhIL-1β stimulation. The issue of SB202190 inhibiting NF-κB transactivation (65) following IL-1β (or TNF-α) cellular activation is probably related to cell context since, in our cell cultures, SB202190 actually had a mild stimulatory effect in this regard.

In summary, this study describes a molecular paradigm in which membrane phospholipid-derived metabolites, released immediately following cytokine cellular activation, modulate cytokine-target gene expression in a fundamental way. It remains to be determined the degree to which target genes are modulated, the type of modulation, and if the phenomenon applies to other lipoidal metabolic pathways and/or cellular phenotypes, allowing the development of a unifying hypothesis in this regard.

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