Elucidation of the signaling network of COX-2 induction in sheared chondrocytes: COX-2 is induced via a Rac/MEKK1/MKK7/JNK2/c-Jun-C/EBPβ-dependent pathway

Zachary R. Healy,* Fei Zhu,* Joshua D. Stull, and Konstantinos Konstantopoulos K. Elucidation of the signaling network of COX-2 induction in sheared chondrocytes: COX-2 is induced via a Rac/MEKK1/MKK7/JNK2/c-Jun-C/EBPβ-dependent pathway. Am J Physiol Cell Physiol 294: C1146–C1157, 2008. First published March 26, 2008; doi:10.1152/ajpcell.00542.2007.—Shear stress is a pathophysiologically relevant mechanical signal in cartilage biology and tissue engineering. Cyclooxygenase-2 (COX-2) is a pivotal proinflammatory enzyme, which is induced by mechanical loading-derived shear stress in chondrocytes. In the present study, we investigated the transcriptional machinery and signaling pathway regulating shear-induced COX-2 expression in human chondrocytic cells. Deletion and mutation analyses of the human cox-2 promoter reveal that the CCAAT/enhancer-binding protein (C/EBP) and activator protein-1 (AP-1) predominantly contribute to the shear-induced cox-2 promoter activity. Supershift assays disclose that C/EBPβ, but not C/EBPα or C/EBPδ, binds to the C/EBP site, whereas c-Jun binds to AP-1. Individual gene knockdown experiments demonstrate the direct regulation of C/EBPβ expression by c-Jun, and the critical roles of both c-Jun and C/EBPβ in shear-induced COX-2 synthesis. Our studies also indicate that Rac and, to a lesser extent, Cdc42 transactivate MEKK1, which is, in turn, responsible for activation of mitogen-activated protein kinase kinase 7 (MKK7). MKK7 regulates c-Jun NH2-terminal kinase 2 activation, which, in turn, triggers the phosphorylation of c-Jun that controls shear-mediated COX-2 upregulation in chondrocytes. Reconstructing the signaling network regulating shear-induced COX-2 expression and inflammation may provide insights to optimize conditions for culturing artificial cartilage in bioreactors and for developing therapeutic interventions for arthritic disorders.

mechanobiology; signal transduction; shear stress; cyclooxygenase; enhancer-binding protein; mitogen-activated protein kinase kinase; c-Jun NH2-terminal kinase

CYCLOOXYGENASE (COX) IS A bifunctional enzyme that catalyzes the biosynthesis of arachidonic acid to form the peroxide prostaglandin (PG) G2 and a peroxidase reaction in which PGG2 is reduced to PGH2. PGH2 is the common precursor of biologically active prostanoids, including PGE2, PGI2, PGD2, PGG2, and thromboxane (45). COX is known to exist in two isoforms: COX-1 and COX-2. Although the genes for COX-1 and -2 are different, the proteins have ~60% amino acid identity, with much higher conservation of active site residues (14), and a similar molecular mass of ~70 kDa. Despite their similar active site structures, products, and kinetics, COX-1 and -2 isoforms display remarkable differences in their regulation and function. COX-1 is constitutively expressed in many cell types and is presumed to be responsible for the synthesis of housekeeping prostanoids that are critical for normal physiological functions such as the regulation of vascular homeostasis, protection of gastric mucosa, and maintenance of renal integrity (28). On the other hand, COX-2 is either absent or minimally expressed in most normal tissues. However, cox-2 mRNA and protein synthesis can be induced in numerous cell types, including chondrocytes, in a time- and dose-dependent manner by oncogenes, growth factors, and cytokines (15, 20, 29, 41, 55). It is thus presumed that COX-2 is primarily responsible for the elevated production of prostanoids at sites of disease and inflammation.

Indeed, aberrant expression of COX-2 protein in articular tissues is an earmark of arthritis (2, 19, 42). Studies in animal models demonstrated that COX-2 expression was detected in inflamed, but not normal, paws from rats with adjuvant-induced arthritis (3, 38, 42). Oral administration of a selective COX-2 inhibitor markedly suppressed COX-2 expression, PGE2 production, paw edema, and inflammatory cell infiltration in the joints (3). Similarly, an anti-PGE2 antibody prevented the development of tissue edema and hyperalgesia (38), thereby suggesting that the major COX-2-derived prostanooid that contributes to inflammation is PGE2 (28, 38). Superinduction of COX-2 activity accompanied by markedly increased levels of PGE2 release has been reported in human osteoarthritis-affected cartilage (2). Increased COX-2 expression has also been observed in human and an animal model of rheumatoid arthritis (19). Clinical studies have documented the efficacy of COX-2-specific inhibitors in osteoarthritis and rheumatoid arthritis (5). However, recent clinical findings also disclose that certain gastrointestinal, renal (10), and, most importantly, cardiovascular (32) side effects occur with COX-2-specific drugs. Thus, elucidation of the cox-2 transcriptional activation in human chondrocytes in response to either chemical or physical stimuli such as shear stress may aid in the identification of alternative therapeutic targets.

The promoter of the cox-2 gene contains a TATA box and several regulatory elements including nuclear factor-κB (NF-κB) sites, a CCAAT/enhancer-binding protein (C/EBP) motif, Sp1 sites, a cyclic AMP response element (CRE) motif, activator protein-1 (AP-1), AP-2, and polyomavirus enhancer activator 3 (PEA-3) sites (43). Different regulatory elements have been demonstrated to regulate cox-2 transcription in various cell types: NF-κB binding sites (18, 49), the C/EBP motif (8, 17, 35, 43, 49), activating transcription factor/CRE

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sequences (35, 46, 47), and PEA-3 site (47). However, data from cox-2 promoter analysis in chondrocytes are very limited. For instance, it has been reported that binding of C/EBPβ and C/EBPδ factors to their cognate sites on the cox-2 promoter is required for stimulation of its activity by IL-1β in rabbit chondrocytes (49). Moreover, NF-κB binding is involved in the IL-1β-induced cox-2 transcriptional regulation (49). Circumstantial evidence that the CRE motif plays a role in regulation of COX-2 in okadaic acid-stimulated human chondrocytes has been provided by electrophoretic mobility shift assays (EMSA) (29). However, as has been appropriately argued in the literature, direct evidence for the involvement of particular cis-elements in the regulation of gene transcription can only be provided by experiments using reporter gene constructs containing native and mutated promoter sequences (45). Most importantly, the signaling pathways regulating COX-2 expression are species-, tissue-, cell-, and stimulus-specific.

In diarthrodial joints, articular cartilage is subjected to mechanical loads during normal daily activity. Elegant modeling studies have documented that chondrocytes of the superficial and transitional zones are exposed to high and low fluid flow, respectively (6, 7), suggesting that fluid flow is a pathophysiologically relevant mechanical signal in cartilage biology. Notably, in cartilage tissue engineering, chondrocytes/cartilage constructs are constantly exposed to fluid shear levels ranging from ~1 to 23.2 dyn/cm² (40, 53). These studies (40, 53) also disclose that hydrodynamic shear affects construct development beyond nutrient transport, presumably by altering intra-cellular signaling pathways in chondrocytes (40, 53).

In this regard, our studies have been directed at delineating in a rigorous manner the regulatory elements located in the 5'-flanking region of the cox-2 promoter region and their cognate trans-acting factors that contribute to the fluid shear-induced expression of COX-2 in human chondrocytic cells. Moreover, we systematically investigate the involvement of MAPK kinases and their upstream signaling molecules in the regulation of COX-2 synthesis using selective, individual gene knockdowns via dominant negative (DN) antisense oligonucleotide or RNA interference technology.

EXPERIMENTAL PROCEDURES

Reporter, plasmids, and constructs. A 5' flanking DNA fragment −891 to +9 (−891/+9) and its deletion mutants of the human cox-2 gene constructed into a promoterless luciferase expression vector pGL3 (57) were generously provided by Dr. Kenneth K. Wu (University of Texas-Houston Medical School). A wild-type human 2.07-kb cox-1 promoter fragment (−2095/+21) cloned into pGL3 basic luciferase expression vector was also donated by Dr. K. K. Wu (48, 58). The phRL-TK control plasmid ligated to Renilla luciferase was purchased from Promega. The wild-type and mutant ERK1 and ERK2 constructs were gifts from Dr. Melanie Cobb (University of Texas, Southwestern Medical Center at Dallas) (56). The DN-p38α mutant [pCMV-Flag-p38 (agf)] was obtained from Dr. Roger Davis (University of Massachusetts Medical School) (54). The Δkinase-Raf-1 was provided by Dr. Ulf Rapp (University of Wurzburg, Wurzburg, Germany) (31). The wild-type CRE-binding protein (CREB) and the nonphosphorylatable mutant CREB-M1 were donated by Dr. Marc Montminy (Salk Institute) (26). DN Rho, DN Rac, and DN Cdc42 constructs were kind gifts from Dr. Denis Wirtz (Johns Hopkins University) (25). The mitogen-activated protein kinase kinase 7 (MKK7) short hairpin RNA (shRNA) vectors pKD-MKK7-v2 and pKD-MKK7-v5 and the negative control vector pKD-NegCon-v1 were purchased from Upstate. Validated small interfering RNA (siRNA) targeting C/EBPβ, MEKK1, as well as the negative control scramble were from Qiagen.

Cell culture and shear stress exposure. Human T/C-28a2 chondrocytic cells were grown (37°C in 5% CO₂) in 1:1 Ham’s F-12/DMEM (BioWhittaker) supplemented with 10% FBS (1, 12). Twenty-four hours before shear stress exposure, T/C-28a2 cells were incubated in serum-free medium containing 1% Nutridoma-SP (Roche) (1, 12), a low-protein serum replacement that maintains chondrocyte phenotype.

T/C-28a2 cells were exposed to shear stress in medium containing 1% Nutridoma by use of a parallel-plate flow chamber with a recirculating flow loop (37°C in 5% CO₂) (1, 13). In select experiments, cells were incubated with pharmacological agents for 2 h before shear exposure.

Transient transfection. T/C-28a2 cells were transfected with ~13 μg of total plasmid using Lipofectamine 2000 (Invitrogen, 30 μl) for 6 h in serum-containing medium. For promoter analysis studies alone, cells were transfected with 12 μg of COX-2/pGL3 reporter vector and 0.8 μg of phRL-TK control vector (15:1 ratio). For cotransfection experiments (e.g., with DN mutants), cells were transfected with 6 μg of the promoter/reporter construct, 6 μg of the additional construct, and 0.4 μg of phRL-TK. Following transfection, cells were incubated for 48 h in serum-free medium containing 1% Nutridoma and were subsequently exposed to the indicated treatments. Transfection efficiency was assessed by flow cytometry using pEGFP-N2 (BD Biosciences). Cotransfection efficiency, defined as the population of cells receiving copies of both plasmids, was determined by flow cytometry with the plasmid of enhanced green fluorescence protein (pEGFP-N2) and the plasmid of enhanced yellow fluorescence protein (pYFP). Approximately 70–75% of the transfected cells expressed significant amounts of both GFP and YFP. For the use of siRNA or antisense RNA, cotransfection efficiency was determined in an analogous manner using pEYFP and a FITC-labeled negative control siRNA (Qiagen).

Preparation of nuclear extract. Following fluid shear exposure, nuclear extracts were collected from slides using the NE-PER nuclear extraction kit (Pierce). Briefly, the slides were washed two times in ice-cold PBS, scraped, and incubated for 20 min in hypotonic buffer, and detergent was added to lyse the cellular membrane. The lysates were incubated for an additional 10 min on ice and vortexed for 15 s, and the nuclei were collected by centrifugation (2 min, 10,000 g, 4°C). The nuclei were lysed for 40 min on ice in a hypertonc buffer and were vortexed for 15 s every 10 min. The specimens were centrifuged (13,000 g, 10 min, 4°C), and the supernatants containing the nuclear extract were stored at −80°C. Protein concentrations were determined by the bichinchoninic acid reagent (BCA, Pierce).

Promoter activity assays. Firefly and Renilla luciferase activities were measured by use of the Dual-Luciferase Report Assay kit (Promega). Firefly luciferase activities were normalized to the Renilla luciferase controls. Data are expressed as ratios of shear to static normalized firefly luciferase activity unless otherwise stated.

EMSA and supershift assay. As shown in Figs. 1 and 2, three 5’-biotinylated oligonucleotide probes were synthesized containing the C/EBP, PEA-3/nuclear factor of activated T cells (NFATc)/AP-1, and CRE cis-elements present in the −159/−48 region of the cox-2 promoter. The PEA-3/NFATc/AP-1 and the CRE probes were partially mutated to destroy overlapping response elements, because these two sites are located in close proximity (Figs. 1 and 2). EMSAs were carried out with a commercially available nonradioisotopic EMSA kit (LightShift Chemiluminescence EMSA kit; Pierce). Briefly, nuclear extracts (4 μg) were incubated in binding buffer [10 mM Tris, 50 mM KCl, 1 mM DTT supplemented with 1 μg poly(dI-dC), 2.5% glycerol, 0.05% NP-40, and 5 mM MgCl₂] containing 12 fmol biotinylated, double-stranded probes for C/EBP, PEA-3/NFATc/AP-1, and CRE for 30 min at 4°C. For competition binding, a 200-fold
excess of unlabeled (cold) probe was incubated with nuclear extracts before the inclusion of the biotinylated one. For supershift assays, the nuclear extracts were preincubated for 30 min at 4°C with anti-CEBPα, anti-C/EBPβ, anti-C/EBPδ (Active Motif), anti-PEA-3 (Santa Cruz), anti-nFATc, anti-CREB-1, anti-phospho-CREB-1, anti-CREB-2 (Santa Cruz), anti-c-Jun, or anti-c-Fos antibodies (Active Motif); the biotinylated oligonucleotide probe specific for C/EBP, PEA-3/nFATc/AP-1, or CRE was then added and incubated for another 30 min at 4°C. The protein-DNA complexes were resolved on a native 6% DNA retardation gel (Invitrogen) in 0.5× Tris-borate-EDTA running buffer (Invitrogen), transferred to a nylon membrane (Pierce), and visualized using the LightShift Chemiluminescence system.

Site-directed mutagenesis. cox-2 promoter (−891/+9)-pGL3 constructs were mutated at selected sites in the cox-2 promoter using the QuickChange Site-directed Mutagenesis Kit (Stratagene) in conjunction with the primer sets listed in Fig. 2. The mutated promoter elements were verified by DNA sequencing.

Quantitative real-time PCR. PCR primers were designed using the Primer3 website (Whitehead Institute, Massachusetts Institute of Technology) (39), and their specificity was examined by National Center for Biotechnology Information Basic Local Alignment Search Tool of the human genome. In the case of multiple splice variants, the conserved regions of the gene of interest were used for primer design. Amplicon specificity was verified by first-derivative melting curve analysis using software provided by Perkin-Elmer/ Applied Biosystems (27). The GenBank accession numbers and forward (F-) and reverse (R-) primers are as follows: c-jun (W91655), F-GCACCG- CCAAACCTAGCCATC and R-TAGGCATAAGCTCGCCCTTC; jnk2 (AA157286), F-CTGGCCCTGACACAGAGACAG and R- CCATCAACTCCCAAGCATTT; cox-2 (AA642111), F-TGAG- CATCTACGGTTTGCTG and R-AACTGCTCATCACCCCATTC; cox-1 (AA454668), F-CTTTCCCTCAAGGGTCTCC, and R-AGG- GACAGGCTTCTGGTGTG; β-actin (XM_004814), F-ATCGGGCG- GCTCCATCC and R-GGGGCACAGAGGCTCATC; and gapdh (AA77488), F-GGCCCTCAAGGATATAGAC and R-AGGGTC- TACATGGCAACT.

Single-stranded cDNAs were generated from reverse transcription of RNA samples using the TaqMan RT kit (Applied Biosystems), diluted, and then subjected to PCR with SYBR Green (Applied Biosystems) as the detected fluorophore. Incorporation of the SYBR Green dye into the PCR products was monitored in real time with the ABI Prism 7900HT sequence detection system. The carboxy-Xrhodamine passive reference dye was used to factor in well and pipetting variability. GAPDH and β-actin were used as “housekeeping” genes. Standard curves were determined for each specimen using the 18S ribosomal RNA (18S rRNA kit; Ambion, Austin, TX) and were used to normalize and quantify mRNA levels via 18S rRNA amplification.

Antisense oligonucleotides. Control and antisense oligonucleotides against c-jun, c-fos, and JNK2 were generously provided by ISIS Pharmaceuticals (1, 4). For analysis of mRNA transcript levels, T/C-28a2 cells were transfected with 400 nM of antisense oligonucleotides using Lipofectamine 2000 (1, 4). For analysis of mRNA transcript levels, T/C-28a2 cells were transfected with 400 nM of antisense oligonucleotides using Lipofectamine 2000 (30 μl) for 6 h in serum-containing media. For studies in which antisense oligonucleotides were cotransfected with reporter plasmids, 400 nM of oligonucleotide and 6 μg of reporter vector, and 0.4 μg of control vector were used. The antisense oligonucleotide targeting JNK2 and a mismatch control oligonucleotide were synthesized as uniform phosphorothioate, chimeric oligonucleotides with 2'-O-methoxymethyl-modified sugars on nucleotides 1–5 and 16–20 and 2'-deoxy sugars on nucleotides 6–15. The antisense oligonucleotides targeting c-jun or c-fos alone and a chemistry control oligonucleotide were synthesized as uniform phosphorothioate oligonucleotides and 2'-deoxy sugars on all nucleotides. The oligonucleotides were synthesized using an Applied Biosystems

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380B automated DNA synthesizer (Applied Biosystems) and were purified as described previously (4). The sequences of the oligonucleotides used in these studies were previously reported (1).

**Intracellular protein staining.** T/C-28a2 cells were fixed with 1.0% formaldehyde for 10 min at 37°C, permeabilized in 90% methanol for 30 min on ice, and incubated at room temperature (RT) for 10 min in blocking buffer (0.5% BSA in Dulbecco’s PBS). Cell specimens were then incubated with monoclonal antibodies specific for JNK1/2 or phospho-JNK1/2, ERK1/2 or phospho-ERK1/2, p38 or phospho-p38, or appropriate isotype controls for 30 min at RT. Next, cells were washed twice in blocking buffer, incubated with FITC-goat anti-rabbit IgG, and analyzed by flow cytometry.

**Western hybridization.** Total protein liberated using a cell lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and protease inhibitor cocktail, pH 7.5) or nuclear extracts from shear-induced and matched static control specimens were separated by 10–14% SDS-PAGE and electrotransferred on polyvinylidene difluoride membrane (Millipore). The membrane was blocked for 1 h in Starting Block solution (Pierce) at RT, incubated with a primary antibody against MEKK1, MKK7, phospho-MKK7, phospho-JNK2, or C/EBPβ for 1 h at RT, washed four times in Tris-buffered saline-Tween 20, and incubated for 1 h with the appropriate horseradish peroxidase secondary antibody (1:5,000; Sigma) at RT. The membrane was washed four times in Tris-buffered saline-Tween 20, and reactive bands were detected using a Super Signal chemiluminescence substrate kit (Pierce). To ensure equal loading of samples in each lane, membranes were stripped and reprobed with a β-actin (total protein) or lamin (nuclear protein) antibody (24, 51).

**RESULTS**

The C/EBP/NF-IL-6 and AP-1 binding sites play a pivotal role in the induction of COX-2 in shear-activated human chondrocytic cells. We have previously reported that fluid shear stress induces COX-2 mRNA synthesis in T/C-28a2 chondrocytic cells in a time- and magnitude-dependent manner (1, 13). As a first step, we examined the effects of shear on COX-2 promoter activity in T/C-28a2 cells transiently transfected with a construct encompassing the 5′-flanking region of the human Cox-2 gene from −891 to +9 bp (−891/+9) cloned into a promoterless luciferase expression vector (43). The aforementioned region contains all previously reported putative regulatory elements responsible for COX-2 induction in diverse cell types activated by distinct stimuli (8, 17, 35, 43, 46, 47, 49). In accord with the time-course induction of COX-2 mRNA synthesis (1), the COX-2 promoter activity increased after T/C-28a2 cell exposure to 20 dyn/cm² for 1 h, and it reached a plateau after 3 h of shear application (Fig. 3A).

Moreover, the COX-2 promoter activity was induced in human chondrocytic cells only when shear stress exceeded the threshold value of 5 dyn/cm² and plateaued at 20 dyn/cm² (Fig. 3B). These data are in concert with our prior findings showing that prolonged exposure of T/C-28a2 cells to 4 or 5 dyn/cm² does not elicit COX-2 mRNA (1) or protein (13) expression. As expected (1), the COX-1 promoter activity was not altered under any of the conditions examined in this work (Fig. 3).

To identify the putative cis-elements on the COX-2 gene promoter responsible for COX-2 induction in shear-activated chondrocytes, T/C-28a2 cells were transiently transfected with a series of human COX-2 promoter-deletion constructs ligated to a promoterless luciferase expression vector pGL3 and were subsequently subjected to either 0 (static) or 20 dyn/cm² for 3 h followed by firefly luciferase activity measurements. To correct for potential differences in transfection efficiency, a phRG-TK control plasmid ligated to Renilla luciferase and the pGL3-firefly luciferase reporter vector under the control of either the human cyclooxygenase (COX)-2 (−891/+9) or COX-1 (−2095/−21 bp) promoter were used to allow for recovery for 48 h. Cells were then subjected to either 0 dyn/cm² (static) or 20 dyn/cm² for the indicated periods of time (A). Alternatively, cells were subjected to prescribed levels of shear for 3 h (B). Following shear exposure, T/C-28a2 cells were lysed, and luciferase activities were determined. The COX-2 and COX-1-driven firefly luciferase activities were normalized to the Renilla luciferase controls. Data represent means ± SD of 4 independent experiments and are expressed as ratios of shear to static firefly luciferase activity.

**Fig. 3.** Effects of shear exposure time (A) and shear stress intensity (B) on COX-2 and COX-1 promoter activity in human chondrocytic cells. T/C-28a2 cells were transfected with the phRL-TK control plasmid ligated to Renilla luciferase and the pGL3-firefly luciferase reporter vector under the control of either the human cyclooxygenase (COX)-2 (−891/+9) or COX-1 (−2095/−21 bp) promoter and were allowed to recover for 48 h. Cells were then subjected to either 0 dyn/cm² (static) or 20 dyn/cm² for the indicated periods of time (A). Alternatively, cells were subjected to prescribed levels of shear for 3 h (B). Following shear exposure, T/C-28a2 cells were lysed, and luciferase activities were determined. The COX-2 and COX-1-driven firefly luciferase activities were normalized to the Renilla luciferase controls. Data represent means ± SD of 4 independent experiments and are expressed as ratios of shear to static firefly luciferase activity.
Fig. 4. Identification of the putative cis-elements on the cox-2 gene promoter responsible for COX-2 induction in human chondrocytic cells subjected to fluid shear. 

A: deletion analysis of luciferase activity of cox-2 gene promoter constructs in T/C-28a2 chondrocytic cells. T/C-28a2 cells were transfected with the pRL-TK control plasmid ligated to Renilla luciferase and the pGL3-luciferase reporter vector under the control of the indicated regions of the cox-2 promoter, allowed to recover for 48 h, and then subjected to either static (0 dyn/cm²) or shear flow (20 dyn/cm²) conditions for 3 h. The cox-2-driven firefly luciferase activity was normalized to the Renilla luciferase control. Data (means + SD) are expressed as absolute values of luciferase activity (firefly/Renilla, n = 4), whereas the numbers to the right of the bars represent the ratio of shear (20 dyn/cm²) to static (0 dyn/cm²) (shear/static) luciferase activity. 

B: analysis of the contributions of individual cis-elements to shear-induced cox-2 promoter activity in human chondrocytic cells by site-directed mutagenesis. The cox-2-pGL3 vector (−891/+9) was subjected to site-directed mutagenesis to generate vectors with inactive C/EBP (−132/−124, mC/EBP), PEA-3/NFATc/AP-1 (−75/−61, mPEA-3/NFATc/AP-1), or CRE (−59/−53, mCRE) response elements. T/C-28a2 cells were transfected with the pRL-TK control plasmid ligated to Renilla luciferase and the indicated pGL3-luciferase reporter vector under the control of either the wild-type or individual site-specific mutated cox-2 promoter (−891/+9), allowed to recover for 48 h, and subjected to either static (0 dyn/cm²) or shear flow (20 dyn/cm²) conditions for 3 h. The cox-2-driven firefly luciferase activity was normalized to Renilla luciferase control. Data (means + SD) are expressed as absolute values of luciferase activity (firefly/Renilla, n = 4), whereas the numbers to the right of the bars represent the ratio of shear (20 dyn/cm²) to static (0 dyn/cm²) (shear/static) luciferase activity.

Cumulatively, these data suggest that two regions (−193 to −120 bp and −96 to −53 bp) contain possible shear stress-response elements. The potential involvement of region −891 to −568 bp in the regulation of cox-2 promoter activity in response to shear is highly unlikely, since deletion of region −182 to −49 bp from the (−891/+9) construct was sufficient to abrogate the shear-induced luciferase activity (Fig. 4A). Of note, the absolute value of luciferase activity of sheared chondrocytes remains nearly constant (−8) upon transfection with constructs (−891/+9, −568/+9, −459/+9, −362/+9, and −193/+9) (Fig. 4A). Rather, the absolute value of firefly luciferase activity of chondrocytes under static conditions increases upon deletion of the −891 to −568 bp region, suggesting that a repressor element may bind this region of the cox-2 promoter under static conditions.

Analysis of the consensus sequence in (−193/−120) and (−96/−53) regions reveals the presence of the following cis-elements: Sp1, AP-2, C/EBP/NF-IL-6, PEA-3, NFATc/AP-1, CRE, and E-box (Fig. 1). To eliminate the possibility that the decreases in shear-induced luciferase activity were attributable to the difference in the length of the promoter region in the plasmids used, we introduced mutations in the presumed response elements in the same length of cox-2 promoter (−891/+9) gene (Fig. 2). Introduction of a mutation into C/EBP/NF-IL-6 (−132/−124) or PEA-3/NFATc/AP-1 (−75/−61) markedly repressed the shear-induced luciferase activity relative to the wild-type reporter, suggesting that these sites are shear stress-response elements (Fig. 4B). In contrast, the introduction of a mutation into the CRE (−59/−53) site did not cause any significant reduction in the ratio of shear to static luciferase activity relative to the wild-type control (Fig. 4B). Taken altogether, our data reveal the critical involvement of C/EBP/NF-IL-6 and PEA-3/NFATc/AP-1 in the regulation of shear-induced COX-2 expression in human chondrocytic cells. C/EBPβ and c-Jun are involved in the regulation of COX-2 induction in shear-activated human chondrocytic cells. Having identified the putative cis-elements on the cox-2 gene promoter responsible for COX-2 induction in shear-activated chondrocytes, our next set of experiments aimed to determine their cognate transcription factors. To this end, EMSAs were carried out using nuclear extracts from static (0 dyn/cm²) and sheared (20 dyn/cm²; 3 h) chondrocytic cell specimens along with three biotinylated double-stranded oligonucleotide probes containing motifs of C/EBP/NF-IL-6, PEA-3/NFATc/AP-1, and CRE on the basis of their consensus sequence in the human cox-2 gene promoter (Fig. 2). When the C/EBP/NF-IL-6 probe was
incubated with nuclear extracts from sheared, but not static, T/C-28a2 chondrocytes, a band indicating their complex was readily detected in the gel (Fig. 5A). The specificity of the shear-induced binding was demonstrated by the addition of a 200-fold excess of nonbiotinylated (cold) C/EBP/NF-IL-6 probe before the inclusion of the biotinylated probe, which prevented the detection the complex formation (Fig. 5A). When the C/EBP/NF-IL-6 probe and sheared nuclear extracts were incubated with an anti-C/EBPβ antibody, a marked supershift of the complex was denoted in the gel (Fig. 5A). In contrast, no supershift was detected with an anti-C/EBPα or an anti-C/EBPδ antibody (Fig. 5A), suggesting that shear stress induced pronounced and selective C/EBPβ binding to the C/EBP/NF-IL-6 binding site. Likewise, fluid shear stress induced the formation of the complex with the nuclear extracts and PEA-3/NFATc/AP-1 probe, which was supershifted after incubation with an anti-c-Jun or anti-c-Fos antibody (Fig. 5B). No supershift was noted with an anti-PEA-3 or anti-NFATc antibody (Fig. 5B).

The presence of a complex was revealed in the gel upon incubation of nuclear extracts from static control chondrocytes with the biotinylated CRE probe (Fig. 5C). Fluid shear did not further increase the binding of the nuclear extracts to CRE (Fig. 5C), as has also been noted for MC3T3-E1 murine osteoblastic cells (35). The shifted band was recognized by an anti-CREB-1, but not anti-phospho-CREB (anti-pCREB-1) antibody. A marked supershift was noted with an anti-C/EBPβ antibody, with the mixtures being then subjected to nondenaturating PAGE (6% polyacrylamide gel). Closed and open arrows indicate shifted and supershifted bands, respectively. N and S, no-shear and shear conditions, respectively.

The genetic intervention did not significantly impair the ratio of COX-2 expression (29). Moreover, supershift assays suggested a possible role for c-Fos in the induction of COX-2 in shear-activated chondrocytes (Fig. 5B). We, therefore, wished to examine its functional involvement using an antisense oligonucleotide inhibiting c-fos transcript. Our data disclose that this genetic intervention failed to significantly alter COX-2 promoter activity in response to shear activation (Fig. 6). In marked contrast, knocking down c/EBPβ by RNA interference repressed shear-induced COX-2 promoter activity by 60% relative to appropriate controls (Fig. 6). Bioinformatics analysis of
the human c/ebpβ promoter region reveals the presence of an intact AP-1 site at −829/−823. To this end, we wished to investigate the potential role of c-Jun in the regulation of C/EBPβ expression in sheared chondrocytes. Immunoblot analysis of nuclear extracts revealed that application of shear stress (20 dyn/cm²) to T/C-28a2 chondrocytic cells upregulated C/EBPβ expression, which was abolished by the presence of an antisense oligonucleotide, but not a control one, inhibiting the c-jun transcript (Fig. 7). Taken altogether, our data disclose that c-Jun directly regulates C/EBPβ expression. Moreover, c-Jun and C/EBPβ are critically involved in the regulation of shear-mediated COX-2 upregulation in human chondrocytic cells.

Shear-activated Rac/MEKK1/MKK7/JNK2 signaling pathway regulates COX-2 expression in chondrocytic cells. We next aimed at elucidating the upstream signaling molecules regulating COX-2 expression in human chondrocytic cells stimulated with fluid shear. As a first step, we confirmed the pivotal role of JNK2 in shear-mediated COX-2 induction, using an antisense oligonucleotide that inhibits the JNK2 transcript. In accord with our previously published data (1), this genetic intervention suppressed by 70% the shear-induced cox-2 promoter activity (Fig. 8). Application of high fluid shear to chondrocytic cells induced not only the phosphorylation of JNK1/2, but also that of ERK1/2 and p38 (Fig. 9). However, it is noteworthy that the phosphorylation of ERK1/2 was transient and was detected only after 1 h of chondrocytic cell exposure to 20 dyn/cm², whereas elevated phospho-JNK1/2 and phospho-p38 were observed at both the 1- and 3-h time points (Fig. 9). In view of these observations, we investigated the potential functional contribution of ERK1/2 and p38 in the regulation of COX-2 induction in sheared chondrocytes. Use of DN mutants specific for ERK1 or ERK2 or p38 failed to significantly repress the shear-mediated cox-2 promoter activity (Fig. 8, B and C). Moreover, overexpression of either ERK1 or ERK2 in chondrocytes did not have any effect on COX-2 induction (Fig. 8B). To eliminate the possibility that the inhibitory efficacy of these genetic interventions may be obscured by the limited transfection efficiency, experiments were also carried out using specific pharmacological inhibitors of ERK1/2 (U-0126) and p38 (SB-203850). In concert with the results from the DN mutant studies, none of these pharmacological agents significantly impaired the shear-induced cox-2 promoter activity (Fig. 8, B and C).

In light of our data demonstrating the critical involvement of the MAPK JNK2 pathway in this process, our next series of experiments was directed at identifying the MAPK kinase (MAPKK), which phosphorylates JNK2. MAPPKs are in turn activated by MAPKK kinases (MAP3Ks), which are serine/threonine kinases. It is now established that JNK activation occurs via either MAPKK7 (MKK7) or MKK4 phosphorylation of JNK Thr or Tyr, respectively (52). Our previously published microarray data revealed an induction of MKK7, but not MKK4, in T/C-28a2 cells subjected to a shear stress level of 20 dyn/cm², with ratios of 3.6 and 8.6 after 1.5 and 24 h of stimulation, respectively (1). Moreover, analysis of gene expression data using clustering algorithms showed that MKK7 and JNK2 were coregulated (1), thereby suggesting that MKK7 may be upstream of JNK2 activation. To assess the potential functional involvement of MKK7 in shear-mediated COX-2 induction, T/C-28a2 chondrocytic cells were transfected using MKK7 shRNA before their exposure to either static or shear conditions. This genetic intervention repressed by 55% the cox-2 promoter activity evoked by high shear (Fig. 8A), which correlates with the maximal transfection efficiency monitored in these particular experiments. The efficacy of this intervention in knocking down the shear-induced MKK7 expression was confirmed by Western blot analysis using an MKK7 specific MAb (Fig. 8D). Immunoblot assays also reveal that MKK7 knockdown markedly suppressed the shear-mediated phosphorylation of JNK1/2 without impairing the expression of MEKK1, which is upstream of MKK7 (Fig. 8D). On the other hand, knocking down MEKK1 by RNA interference resulted in a pronounced reduction of shear-induced phosphorylation of MKK7 (Fig. 8E) and diminished cox-2 promoter activity in response to high shear stress by ~60% (Fig. 8A). Taken together, our data suggest that MEKK1 phosphorylates MKK7, which in turn activates JNK2, which is responsible for the phosphorylation of c-Jun and induction of COX-2 synthesis in shear-activated human chondrocytes.

Activation of the JNK pathway has been reported to involve the small GTP-binding proteins of the Rho family, including RhoA, B, C, Rac1, 2 and Cdc42 in a cell type- and stimulus-dependent manner, which may in turn get activated by either the Ras protooncogene or the phosphatidylinositol 3-kinase (PI3K) pathway (9). Interestingly, previous work has revealed a role for Rho and Raf-1 in the induction of COX-2 synthesis in human mammary epithelial cells treated with either microtubule-interfering agents (46) or transformed with HER-2/neu (erbB-2) (47). In marked contrast with these data (46, 47), the stimulation of cox-2 promoter activity evoked by high fluid shear was not altered by overexpressing DN forms of Rho or Raf (Fig. 10A). To further establish the lack of Rho involvement in this signaling pathway, quantitative real-time PCR experiments were carried out to monitor the effect of the DN Rho mutant on the JNK2, c-jun, and cox-2 mRNA synthesis induced by shear. Our data reveal that this genetic intervention did not interfere with the shear-induced upregulation of JNK2, c-jun, and cox-2 expression (Fig. 10B). On the other hand, use of a DN mutant specific for Rac drastically inhibited shear-induced cox-2 promoter activity (Fig. 10A). Interestingly, knocking down Cdc42 expression also reduced, albeit to a lesser extent, cox-2 promoter induction (Fig. 10A). These genetic interventions were also effective, albeit to different extents, in repressing shear-induced JNK2, c-jun
and cox-2 mRNA synthesis (Fig. 10B), thereby providing further evidence for their involvement in the regulation of shear-induced COX-2 expression. Taken altogether, our data provide direct evidence for the critical involvement of Rac and Cdc42 in the regulation of JNK2/c-Jun-dependent COX-2 induction in sheared activated chondrocytes (Fig. 11). Moreover, MEKK1 and MKK7 appear to lie down-stream of Rac and Cdc42 and directly upstream of the JNK2/c-Jun pathway (Fig. 11).

**DISCUSSION**

COX-2 is a pivotal proinflammatory enzyme, which is induced in chondrocytes by various stimuli in a time- and
dose-dependent manner, and has been implicated in the pathogenesis and progression of arthritic disorders (2, 19, 42). It is now well established that the signaling pathways regulating COX-2 expression are species-, tissue-/cell- and stimulus-specific. Although high fluid shear has been reported to induce COX-2 expression in human chondrocytes (1, 44), the transcription factors and signaling intermediates regulating its synthesis remain largely obscure. In light of compelling evidence suggesting that fluid flow is a pathophysiologically relevant mechanical signal in cartilage biology and tissue engineering (6, 7, 40, 53), we directed our efforts at delineating the signaling network of COX-2 induction by fluid shear in human chondrocytic cells. The major findings of the study are:

1) The C/EBP/NF-IL-6 and AP-1 regulatory elements predominantly contribute to the shear-induced \( \text{cox-2} \) promoter activity;
2) C/EBP\( \beta \) binding to the C/EBP site along with c-Jun binding to the AP-1 motif mediate COX-2 induction in sheared hu-

Fig. 9. Analysis of MAPK phosphorylation under conditions of shear loading. T/C-28a2 cells were exposed to either static conditions (0 dyn/cm\(^2\)) or laminar shear flow (20 dyn/cm\(^2\)) for 1 or 3 h, fixed, and permeabilized. The cells were then incubated with anti-JNK1/2 or anti-phospho-JNK1/2 (p-JNK) (A), anti-ERK1/2 or anti-phospho-ERK1/2 (B), and anti-p38 or anti-phospho-p38 (C). Following incubation with FITC-conjugated goat anti-rabbit IgG, cells were analyzed by flow cytometry. Data are relative to static samples under the same conditions and represent means ± SD (\( n = 3 \)). *\( P < 0.05 \), §\( P < 0.10 \).

Fig. 10. Elucidation of GTPase involvement in COX-2 induction. A: T/C-28a2 cells were cotransfected with the phRL-TK\(^-\) control plasmid ligated to Renilla luciferase (0.4 \( \mu \)g), the pGL3 luciferase reporter vector under the control of the \( \text{cox-2} \) promoter (−891/+9; 6 \( \mu \)g), and pEGFP-null, dominant negative (DN)-Raf, DN-Rho, DN-Rac, or DN-Cdc42. Cells were allowed to recover for 48 h and were then subjected to either static conditions (0 dyn/cm\(^2\)) or laminar shear flow (20 dyn/cm\(^2\)) for 3 h, and luciferase activities were determined. The \( \text{cox-2} \)-driven firefly luciferase activity was normalized to Renilla luciferase. Data represent means ± SD of 3 independent experiments and are expressed as ratios of (sheared treatment) to (static treatment). B: T/C-28a2 cells were transfected with the indicated constructs and subjected to either static or fluid shear (20 dyn/cm\(^2\)) conditions for 3 h, and total RNA was isolated. All values represent transcript ratios for sheared to paired static controls and were quantified by quantitative reverse transcription PCR. Data represent means ± SD (\( n = 3 \)). *\( P < 0.05 \), §\( P < 0.10 \).
man chondrocytes; 3) c-Jun regulates the expression of C/EBPβ; and 4) Rac and to a lesser extent Cdc42 transactivate MEKK1, which is in turn responsible for activation of MKK7, and the JNK2/c-Jun/C/EBPβ-dependent induction of COX-2 in human chondrocytes.

Our studies using a series of human cox-2 promoter-deletion constructs as well as single mutation analysis of the cox-2 promoter reveal that the C/EBP and AP-1 motifs, but neither the proximal nor the distal NF-κB sites, are critically involved in the COX-2 induction in human chondrocytic cells in response to high fluid shear, whereas a minor role for CRE cannot be ruled out. These data are in clear contrast to previous reports showing that NF-κB and C/EBPβ are required for maximal activation of cox-2 promoter in human chondrocytic cells subjected to high shear stress (20 dyn/cm²).

On the other hand, significant similarities and disparities exist in the regulation of shear-induced COX-2 induction in MC3T3-E1 murine osteoblastic (35) and human chondrocytic cells. Most importantly, the C/EBP and AP-1 binding sites contribute to the shear stress-induced cox-2 promoter activity in both cell types (35). Although fluid shear selectively enhanced the binding of phospho-CREB to the CRE motif of the cox-2 promoter in murine osteoblastic cells (35), no such interaction was detected in shear-activated chondrocytic cells. Along these lines, introduction of a mutation into the CRE (−59/−53) site did not cause any significant reduction in the cox-2 promoter activity (i.e., ratio of shear to static luciferase activity) relative to the wild-type control in chondrocytic cells, although it had a clear inhibitory effect on murine osteoblastic cells (35). In light of previous reports showing increased binding of c-Jun to the CRE site of the human cox-2 promoter in HER-2/neu-transformed mammary epithelial cells (47) and Rous sarcoma virus-transformed fibroblasts, we examined whether fluid shear induces c-Jun binding to CRE in chondrocytic cells. However, our EMSA experiments did not disclose such an interaction. It is also noteworthy that although the kinetics of COX-2 induction in murine osteoblastic and human chondrocytic cells is similar, the former respond to fluid shear even at a wall shear stress level of 0.18 dyn/cm² (35). COX-2 is also rapidly induced in human umbilical vein endothelial cells subjected to a stress level of 1 dyn/cm² (17), whereas COX-2 induction is detected in human chondrocytic cells only above the threshold stress level of 5 dyn/cm². The enhanced mechanosensitivity of endothelial cells compared with chondrocytes may be related in part to the physiological shear environment (1–4 dyn/cm²) that these cells encounter in vivo. Moreover, the role of COX-2 and its products (e.g., prostaglandins) may be distinct in different tissues. For instance, prostaglandins have anabolic effects on proliferation and differentiation of bone-forming cells, and selective inhibition of COX-2 activity has been reported to inhibit mechanical loading-induced bone formation in rats (11). In contrast, overexpression COX-2 in articular tissues is an earmark of arthritis (2, 19, 42) associated with increased numbers of apoptotic chondrocytes (13, 33, 34, 37). Thus, expression of COX-2 in response to low levels of fluid shear would offer an unfavorable phenotype for chondrocytic cells. Hence, our data demonstrating the requirement of an elevated shear stress threshold for the induction of COX-2 expression suggest that abnormally high mechanical loading is necessary to potentially elicit COX-2-mediated inflammation and cartilage degradation within articular joints.

Numerous studies in diverse cell types (46, 47, 55) including chondrocytic cells (21, 22, 34, 49, 50) have shown that the expression of COX-2 can be altered by changes in MAPK activity. To keep this discussion focused, only pathways that mediate COX-2 induction in chondrocytes will be outlined here. Evidence for the involvement of p38 in COX-2 expression in chondrocytes stimulated with either IL-1β or a nitric oxide (NO) donor was provided by experiments using a DN p38 kinase (50), overexpression of p38 active kinase (50), or specific pharmacological inhibitors (22, 34, 49, 50). The dependence of COX-2 expression in NO-treated chondrocytes on ERK1/2 signaling was documented through the use of ERK1/2 specific inhibitors (22, 34). It is noteworthy that this pharmacological intervention partially suppressed COX-2 expression (22) but abolished PGE₂ production (22, 34), probably by blocking COX-2 activity. Although application of high fluid shear induces the phosphorylation of p38 and ERK1/2, neither
of these kinases mediates the genesis of COX-2 synthesis in shear-activated human chondrocytic cells. In fact, we previously demonstrated and confirmed herein the pivotal role of a JNK2-dependent pathway in the regulation of COX-2 expression in chondrocytes subjected to high shear by using an antisense JNK2-specific oligonucleotide (1, 13). Whereas shear-induced COX-2 synthesis proceeds via an NF-κB-independent mechanism, NF-κB has been implicated in the regulation of COX-2 in NO donor-treated rabbit chondrocytes (21). NO-induced NF-κB activation is negatively regulated by PKCα and PKCζ (21), the latter of which is in turn regulated by p38 kinase (23).

Activation of the JNK pathway has been reported to involve the small GTP-binding proteins of the Rho family, including RhoA, B, C, Rac1, 2 and Cdc42 in a cell-type- and stimulus-dependent manner, which may in turn get activated by either the Ras protooncogene or the PI3K pathway (9). Although a role for Rho has been suggested in the regulation of COX-2 expression in sheared murine osteoblastic cells (36), and in mammmary epithelial cells treated with microtubule-interfering agents (46), use of a DN mutant specific for Rho failed to impair the shear-induced upregulation of JNK2, c-Jun, and COX-2. Similarly, although Raf regulates COX-2 expression in HER-2/neu-positive breast cancer cells, it does not appear to contribute to shear-induced COX-2 synthesis. On the other hand, serial, selective gene knockdown experiments via the use of DN mutants or RNA interference technology illustrate that Rac and, to a lesser extent, Cdc42 regulate MEKK1, which is responsible for the transactivation of MKK7 that lies directly upstream of JNK2, which in turn phosphorylates c-Jun. c-Jun in coordination with C/EBP, which is directly regulated by c-Jun, mediates the genesis of COX-2 synthesis.

Taken altogether, our data suggest that the synthesis of COX-2 expression in chondrocytes subjected to high shear stress is regulated by a Rac/MEKK1/MKK7/JNK2/c-Jun-C/COX-2 expression in chondrocytes subjected to high shear by using an antisense pathway regulating cartilage inflammation in response to high shear may identify potential therapeutic targets for controlling arthritic pathogenesis and/or progression and may be useful in the design of bioreactors for cartilage culture.

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