

Shear-induced Cyclooxygenase-2 via a JNK2/c-Jun-dependent Pathway Regulates Prostaglandin Receptor Expression in Chondrocytic Cells*[§]

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Using cDNA microarrays coupled with bioinformatics tools, we elucidated a signaling cascade regulating cyclooxygenase-2 (COX-2), a pivotal pro-inflammatory enzyme expressed in rheumatic and osteoarthritic, but not normal, cartilage. Exposure of T/C-28a2 chondrocytic cells to fluid shear results in co-regulation of c-Jun N-terminal kinase2 (JNK2), c-Jun, and COX-2 as well as concomitant downstream expression of prostaglandin receptors EP2 and EP3a1. JNK2 transcript inhibition abrogated shear-induced COX-2, EP2, and EP3a1 mRNA up-regulation as well as c-Jun phosphorylation. Functional knock-out experiments using an antisense c-Jun oligonucleotide revealed the abolition of shear-induced COX-2, EP2, and EP3a1, but not JNK2, transcripts. Moreover, inhibition of COX-2 activity eliminated mRNA up-regulation of EP2 and EP3a1 induced by shear. Hence, a biochemical pathway exists wherein fluid shear activates COX-2, via a JNK2/c-Jun-dependent pathway, which in turn elicits downstream EP2 and EP3a1 mRNA synthesis.

Cyclooxygenase (COX)¹ is a critical proinflammatory enzyme that converts arachidonic acid to prostaglandins that have been implicated in the pain and inflammation of rheumatic disease (1). COX is known to exist in two isoforms, COX-1 and COX-2, with similar sequence identities (1, 2). Although the two isoforms have analogous active site structures, catalytic mechanisms, products, and kinetics, they exhibit differences in their regulation and function (1). COX-1, is constitutively expressed in many tissues and cell types and is presumed to be responsible for the synthesis of “housekeeping” prostaglandins that are critical for normal physiological functions (1). However, COX-1 has been reported to be differentially regulated in endothelial cells stimulated with fluid shear (3) or phorbol

12-myristate 13-acetate (4), as well as in mouse osteoblastic MC3T3 cells treated with basic fibroblastic growth factor (5). Nevertheless, a variety of chemical stimuli, such as interleukin-1 α has failed to regulate COX-1 in chondrocytic cells (6).

On the other hand, COX-2 is expressed in few tissues under basal conditions. However, COX-2 mRNA and protein synthesis are induced in a time- and dose-dependent manner in inflammation models such as cytokine-stimulated human macrophages (7) and rat mesangial cells (8). Moreover, in chondrocytic models, COX-2 is regulated by chemical agonists such as okadaic acid via activator protein-1 and cAMP-response element (CRE) binding proteins (9), as well as nitric oxide via extracellular signal-related protein kinase 1/2 and p38 kinase (10), and tumor necrosis alpha via nuclear factor- κ B (11). Thus, several discrete signaling pathways have been implicated in the genesis of COX-2 synthesis that are dependent on the stimulus imposed on the cell. Although fluid shear has been reported to induce COX-2 expression in endothelial (3, 12–14) and osteoblastic cells (15), its effect on chondrocytic cells remains to be investigated. Moreover, the transcription factors and signaling intermediates regulating COX-2 expression in response to fluid shear have not been previously elucidated in any cell type.

COX inhibitors have been extensively used in the treatment of rheumatoid arthritis. Of the various prostanoids, prostaglandin E₂ (PGE₂) is thought to play a key role in the erosion of cartilage and juxta-articular bone. The biological actions of PGE₂ are mediated through its binding to specific G-protein-coupled cell surface prostaglandin EP receptors. There are at least four subtypes of EP receptors, termed EP1, EP2, EP3, and EP4, that directly modulate intracellular levels of inositol phosphate or cAMP (16). The significance of the prostaglandin EP receptors was recently documented in a mouse model of experimentally induced arthritis (17). COX-1, but not COX-2, has been implicated in the regulation of the expression of the prostaglandin receptors in cervical carcinomas (18). On the other hand, inactivation of COX-2 has been reported to increase EP3 and EP4 receptor expression in a murine kidney cell line (19), although it failed to interfere with it in an osteogenic cell line (20). Nevertheless, the effects of mechanical stimuli and COX activity on the regulation of prostaglandin receptors in chondrocytic cells have yet to be examined.

Evidence suggests that abnormal mechanical loading of cartilage may be detrimental to the tissue. Pressure gradients generated from mechanical loading during daily activities drive interstitial fluid movement within the cartilage tissue, suggesting that fluid shear is a pathophysiologically relevant mechanical signal in cartilage metabolism. In this regard, our studies

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¹ The abbreviations used are: COX, cyclooxygenase; CRE, cAMP-response element; PGE₂, prostaglandin E₂; JNK, c-Jun N-terminal kinase; RPA, ribonuclease protection assay; DPBS, Dulbecco’s phosphate-buffered saline; RT, room temperature; AP, activator protein; MKK, mitogen-activated protein kinase kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

have been directed at examining the effects of shear flow on the regulation of COX-1 and COX-2 expression in chondrocytic cells, as well as elucidating potential upstream and downstream pathways, using microarray technology and computational analysis in conjunction with traditional molecular biology techniques. Our findings show that high shear stress (20 dyn/cm²) activates the signaling molecule c-Jun N-terminal kinase 2 (JNK2), which then triggers the phosphorylation of the transcription factor c-Jun. These signaling events are involved in the shear-induced up-regulation of COX-2 at the mRNA and protein levels. Given that COX-1 is not regulated by fluid shear, we show, through the use of a specific COX-2 inhibitor, that COX-2 activity ultimately stimulates the synthesis of the prostaglandin receptor subtypes EP2 and EP3a1.

MATERIALS AND METHODS

Cell Culture—The human T/C-28a2 chondrocytic cell line (21–23) was chosen as a model system for monitoring chondrocytic cellular responses to fluid shear for three important reasons. First, the T/C-28a2 cells have been extensively characterized and shown to behave much like primary human chondrocytes when cultured under appropriate conditions (21–23). Second, a sufficient number of primary human chondrocytic cells to perform microarray and ribonuclease protection assay (RPA) experiments are difficult to obtain from a single operative procedure, whereas the chondrocytic phenotype is gradually lost in primary cultures over serial passaging. Finally, the inherent genetic variation in human chondrocyte cultures prepared from different cartilage donors could make reproducibility an issue. T/C-28a2 cells were cultured in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (Cambrex Bio Science Walkersville, Inc.), supplemented with 1× antibiotic-antimycotic (Invitrogen) and 10% fetal bovine serum (Paragon Biotech), and propagated for a maximum of 10 passages. Before shearing experiments, T/C-28a2 cells were detached from tissue culture flasks by mild trypsinization, then seeded (1×10^6 cells/ml) on 75 × 38-mm glass slides (Corning Glass) and incubated overnight at 37 °C in 10% fetal bovine serum-containing medium. 24 h before shear stress exposure, T/C-28a2 cells were washed with Dulbecco's phosphate-buffered saline lacking Ca²⁺/Mg²⁺ (DPBS), and incubated in serum-free medium composed of 1:1 Ham's F-12 and Dulbecco's modified Eagle's media, supplemented with 1× antibiotic-antimycotic and 1% Nutridoma SP (Roche Molecular Biochemicals) to maintain chondrocytic phenotype (23). Throughout cell culturing and shearing experiments, the T/C-28a2 cells maintained their polygonal morphology and failed to exhibit Type I collagen expression, both of which are congruent with the chondrocyte phenotype (21).

Shear Stress Exposure—Confluent monolayers of T/C-28a2 cells were exposed to shear stress (4, 10, or 20 dyn/cm²) for predefined periods of time by using a parallel plate flow chamber placed in a flow loop gassed with 95% air and 5% CO₂, and enclosed within a 37 °C convective air incubator (24, 25). Serum-free media containing 1% Nutridoma was circulating in the shear apparatus for the duration of the experiment. As a control, T/C-28a2 cells were placed on flow chambers, but no shear flow was generated. These matched static specimens were otherwise exposed to the same conditions as their shear analogs.

RNA Isolation—RNA from sheared specimens and their matched static controls was immediately isolated using TRIzol reagent (Invitrogen). T/C-28a2 cells from four independent flow chambers were pooled together to generate a sheared (or static) sample for either RPA or microarray analysis. RNA was quantified using UV spectrophotometry at the A of 260 nm, and stored at –80 °C until assayed.

Ribonuclease Protection Assay—RPA was used to quantify the relative mRNA expression levels for a selected number of genes at the end of shear flow exposure compared with static controls. Clones for c-Fos, JNK2, c-Jun, COX-1, COX-2, prostaglandin receptors EP3a1 and EP2, and the housekeeping gene GAPDH (Research Genetics) were digested at appropriate restriction sites to serve as *in vitro* transcription templates. Antisense RNA probes were generated using either T3 or T7 polymerase (Promega) in the presence of [³²P]UTP (ICN Radiochemicals), and allowed to hybridize overnight at 55 °C to 10 μg of total RNA isolated from the shear and static specimens. The RNA:RNA duplexes were digested with RNase at 30 °C for 1 h (BD Biosciences Pharmingen). RNase was inactivated by incubating with proteinase K (BD Biosciences Pharmingen) at 37 °C for 20 min, and extracted using phenol/chloroform/isoamyl alcohol (Invitrogen). The mixture was ethanol precipitated, and RNA fragments were resolved using a denaturing

5% monomer (19:1 acrylamide/bisacrylamide) gel (National Diagnostics). The gel was dried, exposed to film, and bands of interest were subjected to densitometric analysis.

Probe Generation and Purification for Microarray Experiments—10 μg of total RNA were reversed transcribed in a mixture containing 6 μg of random hexamers (Invitrogen), 0.01 M dithiothreitol (Invitrogen), 1× aminoallyl-dNTP mixture (25 mM concentrations of dATP, dCTP, and dGTP, 15 mM dTTP, and 10 mM aminoallyl-dUTP) (Sigma), 1× reaction buffer, and 400 units of SuperScript II reverse transcriptase (Invitrogen) at 42 °C overnight. RNA template was then hydrolyzed by adding NaOH and EDTA to a final concentration of 0.2 and 0.1 M, respectively, at 70 °C for 15 min. Unincorporated aminoallyl-dUTP was removed using a Qiagen QIAquick column (26). The probe was eluted using a phosphate elution buffer (4 mM KPO₄, pH 8.5, in ultrapure water), dried, and resuspended in 0.1 M carbonate buffer (26). To couple the aminoallyl with fluorescent labels, NHS-Cy3 (in static control samples) or NHS-Cy5 (in sheared specimens) (Amersham Biosciences) was added in the dark at room temperature (RT) for 1 h. Uncoupled label was removed using the QIAquick column (26).

Microarray Hybridization, Normalization, and Analysis—Aminolane-coated microscope slides printed with a set of 32,448 expressed sequence tags were prehybridized in 5× SSC (Invitrogen), 0.1% SDS (Invitrogen), and 1% bovine serum albumin (Sigma) at 42 °C for 45 min (26). Subsequently, the slides were washed at RT with deionized water, dipped in 100% isopropanol at RT, and allowed to dry. Equal volumes of the Cy-3 and Cy-5 labeled probes were combined and supplemented with 20 μg each of COT1-DNA and poly(A)-DNA (26). This mixture was heated to 95 °C for 3 min, after which an equal volume of hybridization buffer, composed of 50% formamide (Roche), 10× SSC, and 0.2% SDS, was added. The probes were added to the microarray slide and allowed to hybridize at 42 °C overnight. Subsequently, the slide was sequentially washed in solutions containing 1× SSC and 0.2% SDS at 42 °C, 0.1× SSC and 0.2% SDS at RT, and 0.1× SSC at RT, each for 4 min (26), then air-dried and scanned using the ScanArray 3000 (GSI Lumonics).

Expression ratios from individual genes were extracted using TIGR Spotfinder, and normalized with the total intensity algorithm of the TIGR multiexperiment viewer (26). Three independent experiments were performed for each condition, thereby permitting an analysis that satisfies conservative statistical criteria. Genes with a ratio of measured Cy5-to-Cy3 intensities of ≥2.0 for each of the three experiments were considered positively regulated by shear stress, whereas those with a ratio of ≤0.5 were regarded as negatively regulated (12).

After data normalization, average linkage hierarchical clustering analysis with a Euclidean distance metric was performed using the TIGR multiexperiment viewer (26), in which genes are iteratively grouped together based on their distance metric. Alternatively, clustering analysis was performed using self-organizing maps (Euclidean distance metric, 3 × 3 hexagonal topology) (27), in which a neural network is trained via competitive learning and subsequently used to cluster the gene expression data. Both clustering algorithms were executed on a data set containing elements that had a fluorescence value of at least 500 to eliminate points suffering from poor hybridization or spotting that may have potentially confounded the clustering output and interpretation.

Western Hybridization—T/C-28a2 cells, from sheared and matched static control specimens, were harvested by mild trypsinization, and washed with DPBS. Total protein was liberated using a cell lysis buffer (10 mM NaPO₄, 2 mM EDTA, 10 mM NaN₃, 120 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, and protease inhibitor) (28), separated by 10% SDS-PAGE, and electrotransferred on PVDF membrane (Millipore). The membrane was blocked overnight in 5% blocking solution (Bio-Rad) at 4 °C, incubated with a primary antibody against JNK2, COX-1, COX-2, c-Jun, or phospho-c-Jun (1:300; Santa Cruz Biotechnology) for 3 h at RT, washed five times in Tris-buffered saline-Tween 20, and incubated for 1 h with a goat anti-rabbit horseradish peroxidase secondary antibody (1:3000; Sigma) at RT. The membrane was washed three times in Tris-buffered saline-Tween 20, and reactive bands were detected using a Super Signal chemiluminescent substrate kit (Pierce). To ensure equal loading of samples in each lane, membranes were stripped and reprobed with a β-actin antibody (1:300; Santa Cruz Biotechnology).

Inhibition Studies—To inhibit viable JNK2, c-Jun, or c-Fos transcript, an antisense oligonucleotide specific for JNK2, c-Jun, or c-Fos (Isis Pharmaceuticals) was transfected into the T/C-28a2 cells. Briefly, confluent T/C-28a2 cells were washed and incubated with 400 nM c-Fos, JNK2, or c-Jun antisense oligonucleotide with Lipofectin reagent (Invitrogen) in 1 ml of serum-free medium. After 5 h, 1 ml of medium supplemented with 2% Nutridoma was added. Cells remained in a 95%

air/5% CO₂ atmosphere for 24 h until they were exposed to shear flow or stationary conditions. β -Galactosidase staining was used to verify transfection efficiency. The antisense oligonucleotide targeting JNK2 and a mismatch control oligonucleotide were synthesized as uniform phosphorothioate, chimeric oligonucleotides with 2'-*O*-methoxyethyl-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 380B automated DNA synthesizer (Applied Biosystems) and purified as described previously (29). The sequences of the oligonucleotides used in these studies are as follows: JNK2 antisense 5'-GCT-CAGTGGACATGGATGAG-3'; JNK2 control, 5'-GCACATTGCACGTG-AATTAC-3'; c-Jun antisense, 5'-TCAGCCCCGACGGTCTCTC-3'; c-Fos antisense, 5'-AAGTCCTTGAGGCCACAGC-3'; c-Jun/c-Fos control, 5'-GTGCGCGAGCCCCGAAATC-3'. To inhibit COX-2 enzyme activity, the specific inhibitor NS-398 (Cayman Chemical) was used at a concentration of 30 μ M (30) within the flow media for the entire duration of the shear experiment as well as for their respective matched static control specimens.

Immunofluorescence Analysis—T/C-28a2 cells on glass slides from sheared specimens and their matched static controls were washed with DPBS, and fixed for 2 min in either ice-cold pure acetone or methanol. Thereafter, specimens were washed in DPBS and incubated with 10% normal blocking serum for 20 min. Cells were then incubated with COX-2 antibody (2 μ g/ml; Oxford Biomedical) in 1.5% normal blocking serum for 1 h at 37 °C and subsequently underwent three 5-min washes with DPBS. A fluorescein-conjugated secondary antibody was added for 45 min at RT, followed with three washes in DPBS. Samples were then mounted using antifade mounting medium and examined using confocal microscopy.

RESULTS

To investigate chondrocytic cellular responses to fluid shear, T/C-28a2 chondrocytic cells were exposed to a stress level of 20 dyn/cm² (representative of a high shear environment, as evidenced in previous studies (31–33)) for prescribed periods of time using a parallel-plate perfusion chamber placed in a flow loop. Microarray analysis revealed that 301 known genes responded to shear stress after a 24-h chondrocytic cell shear exposure, of which 188 were up-regulated and 113 were down-regulated compared with matched static controls.² Shorter shear exposure times (1.5 h) resulted in the up- and down-regulation of 34 and 30 known genes, respectively, some of which are listed in Fig. 1. Using an average linkage hierarchical clustering algorithm with Euclidean distance metric (TIGR multiexperiment viewer program), we determined that JNK2, c-Jun, and COX-2 are contained within the same subtree structure (supplemental Fig. 1a), thereby implicating co-regulation among the three genes upon T/C-28a2 cell exposure to fluid shear. Furthermore, this analysis revealed that the prostaglandin EP2 and EP3a1 receptors, which may act as potential downstream targets of the COX activity, are co-regulated although they reside in a subtree structure different from the one mentioned above (supplemental Fig. 1a). The gene expression data were also analyzed using self-organizing maps (Euclidean distance metric, 3 \times 3 hexagonal topology). The results from this analysis indicate that JNK2, c-Jun, COX-2, and both prostaglandin EP2 and EP3a1 receptors are contained within the same cluster (supplemental Fig. 1b). Moreover, hierarchical analysis within the aforementioned cluster reveals the existence of distinct subtree structures in which JNK2, c-Jun, and COX-2 are tightly grouped together, separately from the prostaglandin EP2 and EP3a1 receptors (supplemental Fig. 1b). Cumulatively, these results indicate that the clustering pattern of the selected genes is conserved irrespective of the algorithm implemented. These microarray clustering data

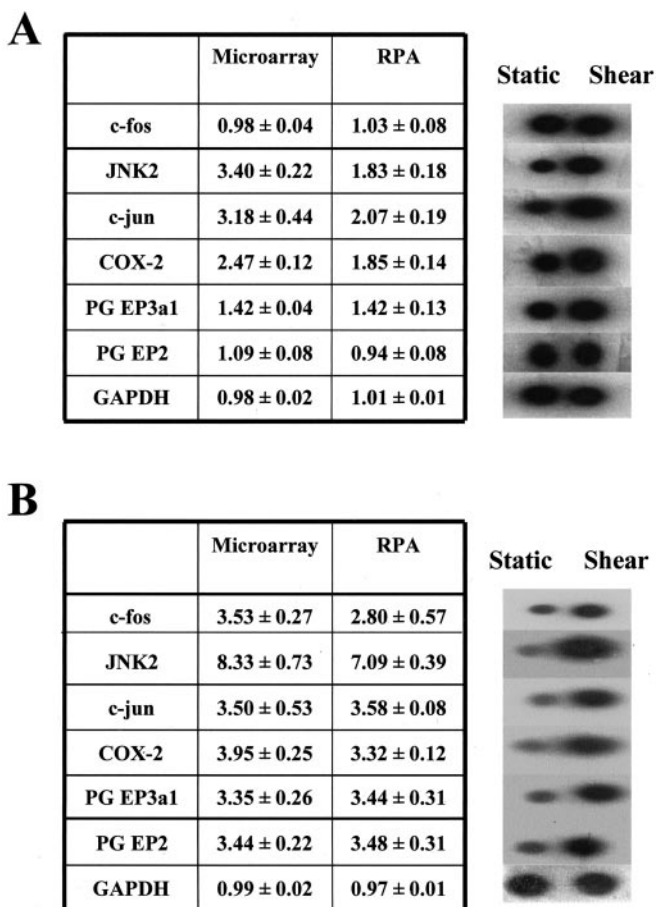


FIG. 1. Validation of the microarray technique with an RPA. Comparison of cDNA microarray intensity ratios (shear/static) and RPA densitometry ratios (normalized to GAPDH) for seven genes, assessed at the 1.5- (A) and 24-h (B) time-points. Values are mean \pm S.E. from three independent experiments. RPA gels show representative bands on each gene for static (control) and shear stress (20 dyn/cm²) conditions.

prompted us to hypothesize that fluid shear activates COX-2 via a JNK2-dependent pathway involving phosphorylation of c-Jun, which in turn elicits downstream EP2 and EP3a1 mRNA synthesis.

As a first step, we examined the accuracy of the microarray gene expression profiling by performing an RPA analysis on the aforementioned genes. Fig. 1 shows microarray ratios, RPA ratios, and RPA autoradiographs on these selected genes at both 1.5 and 24 h. The results from the RPA analysis are in very good agreement with the microarray data, thus validating our microarray procedure and analysis. We next wished to systematically investigate whether up-regulation of JNK2, c-Jun, and COX-2 precedes that of EP2 and EP3a1. To this end, an RPA time-course experiment was performed at 0.75, 1.5, 3, 6, 12, 16, and 24 h. As shown in Fig. 2A, the mRNA expression levels of JNK2, c-Jun, and COX-2 increased after exposure of chondrocytic cells to shear stress for 1.5 h and also remained elevated at the 24-h time point. In marked contrast, significant up-regulation of EP2 and EP3a1 mRNA expression occurred only after 24 h of shear exposure (Fig. 2B), and ensued COX-2 protein expression, which was detected at the 16-h time point (Fig. 3A). No COX-2 protein was detected at earlier time points in both static and sheared samples (Fig. 3A). Subsequent experiments aimed to determine the localization of COX-2 protein after the application of shear stress, using immunofluorescence analysis. In accord with the Western blotting data, COX-2 was absent from T/C-28a2 chondrocytic cells subjected to static

² J. P. Abulencia, Z. R. Healy, and K. Konstantopoulos, unpublished observations.

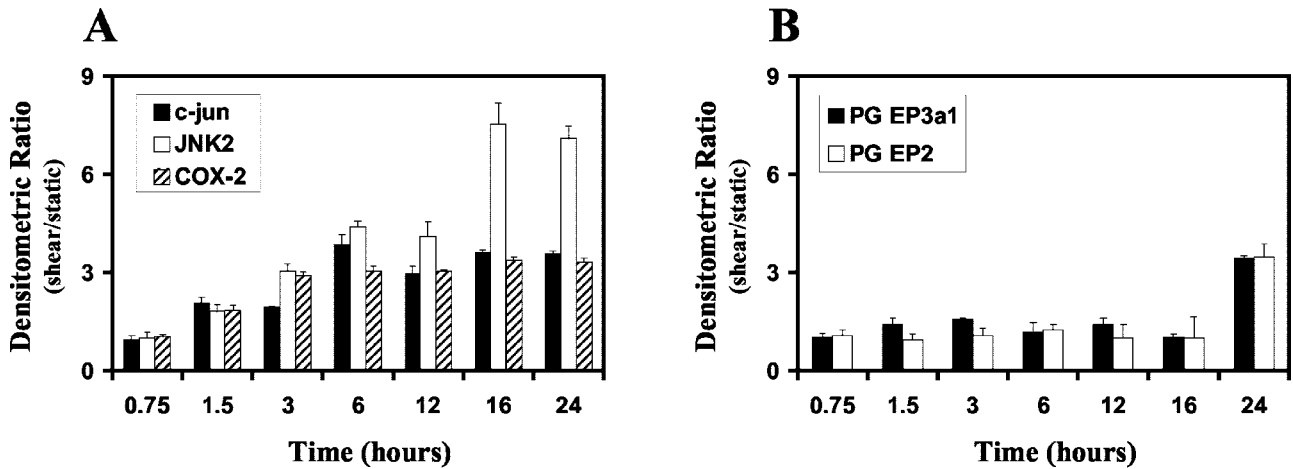


FIG. 2. Effects of shear exposure time on the mRNA expression of JNK2, c-Jun, and COX-2 (A) as well as prostaglandin EP3a1 and EP2 receptors (B). T/C28a2 cells were sheared at 20 dyn/cm² for the indicated periods ranging from 0.75 to 24 h. In static (control) experiments, T/C28a2 cells were exposed to 0 dyn/cm² for 0.75 to 24 h. Values are mean ± S.E. for RPA densitometry ratios (shear/static) from three independent experiments.

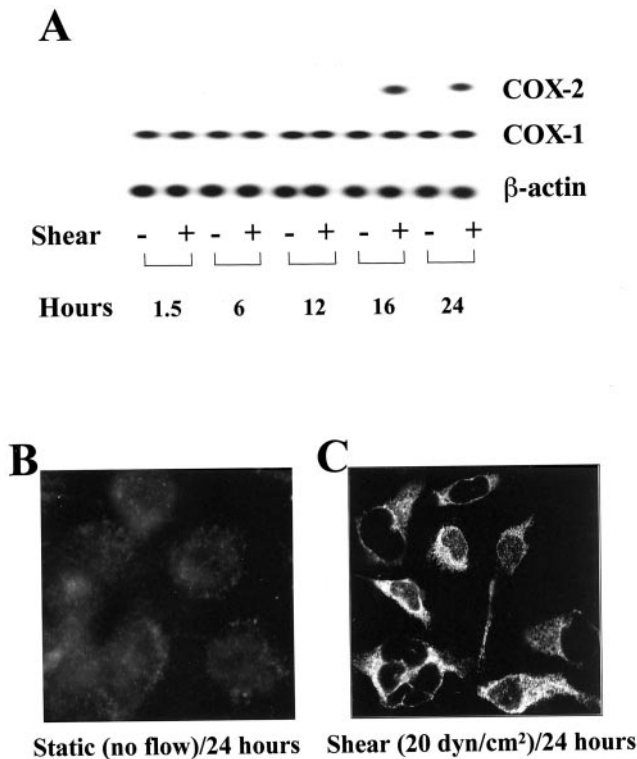


FIG. 3. A, effects of shear exposure time on COX-1 and COX-2 protein levels in T/C28a2 chondrocytic cells. Cells were exposed to shear stress (20 dyn/cm²) for durations ranging from 0.75 to 24 h. The constitutively expressed “housekeeping” protein β-actin was probed as a loading control. COX-2 localization as assessed by immunofluorescence in T/C28a2 chondrocytic cells in the absence (B) or presence (C) of shear stress (20 dyn/cm², 24 h).

(no-flow) conditions for 24 h (Fig. 3B). However, COX-2 staining was evident in chondrocytic cells sheared for 24 h at 20 dyn/cm² and observed to be present in the cytosol and nuclear envelope of shear-stimulated cells (Fig. 3B).

To demonstrate the involvement of the JNK2 pathway in the shear-induced regulation of COX-2, an antisense oligonucleotide inhibiting the JNK2 transcript was transfected into T/C-28a2 chondrocytic cells before their exposure to a shear stress level of 20 dyn/cm² for 24 h. The results indicate that JNK2 inhibition abrogated shear-induced COX-2 expression at both the protein (Fig. 4A) and mRNA (Fig. 5, A and B) levels. In

distinct contrast, a control oligonucleotide failed to affect shear-induced COX-2 expression (Fig. 4A). To verify the efficacy of the antisense JNK2-specific oligonucleotide, Western immunoblot (Fig. 4B) and RPA (Fig. 5, A and B) analyses were carried out showing that it abrogated shear-induced JNK2 protein and mRNA up-regulation, whereas the control oligonucleotide had no effect (Fig. 4B). Cumulatively, these data suggest that the JNK2 represents an upstream signaling element of the COX-2 expression in response to fluid shear.

Among JNKs, JNK2 has been shown to exhibit the highest affinity for c-Jun, and to possess a putative loop region that interacts with the JNK-docking site on c-Jun (34). We therefore examined the effects of shear-induced JNK2 activity on c-Jun phosphorylation in chondrocytic cells. Western blot analysis revealed that application of high shear stress (20 dyn/cm²) to T/C-28a2 cells induced phosphorylation of c-Jun, which was abrogated by the presence of an antisense oligonucleotide (but not a control oligonucleotide) inhibiting the JNK2 transcript (Fig. 4B). To further examine the role of c-Jun in this pathway, an antisense oligonucleotide directed against c-Jun was transfected into T/C-28a2 chondrocytic cells before their exposure to shear (20 dyn/cm² for 24 h). The results indicate that antisense oligonucleotide functional knockout of c-Jun abrogated shear-induced COX-2 mRNA expression levels but left intact the upstream JNK2 transcript (Fig. 5, A and B). In distinct contrast, a control oligonucleotide failed to affect shear-induced COX-2 expression (data not shown). Previous work on chondrocytic cells stimulated with okadaic acid suggested a role for the transcription factor c-Fos in the regulation of COX-2 expression (9). We therefore wished to examine its involvement in the induction of COX-2 in chondrocytic cells subjected to shear. Microarray clustering analysis indicates that c-Fos is not contained in the same sub-tree structure with JNK2, c-Jun, and COX-2, suggesting that it is not likely to be involved in this pathway. This was corroborated by functional knockout experiments, using an antisense c-Fos-specific oligonucleotide, showing that it failed to affect shear-induced COX-2 expression (Fig. 5, A and B). Evidence for the transfection efficiency and functionality of the antisense oligonucleotide to c-Fos is provided by RPA analysis demonstrating that cells transiently transfected with the antisense oligomer, but not a control oligomer, exhibited abolition of shear-induced c-Fos up-regulation (Fig. 5, A and B). Taken together, these data lead us to propose that a JNK2-dependent pathway involving c-Jun phosphorylation regulates shear-induced COX-2 expression. It is noteworthy

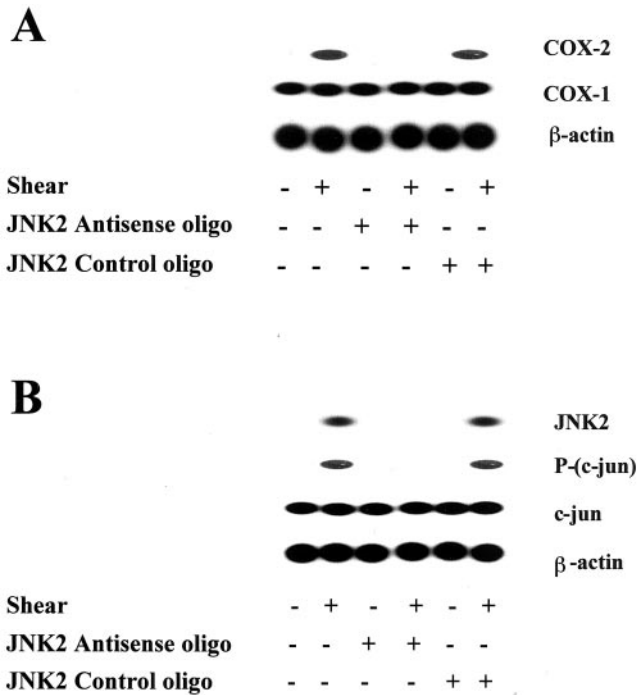
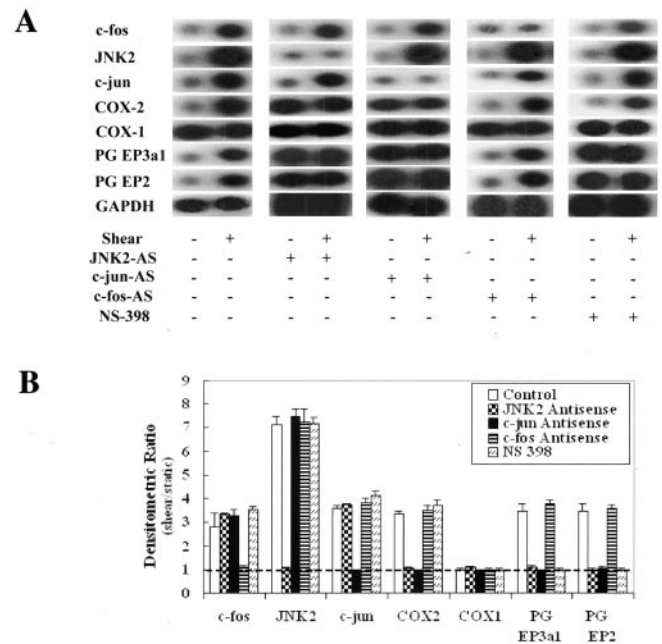


FIG. 4. Effects of JNK2 transcript inhibition on COX-1, COX-2, JNK2, phosphorylated c-Jun, and total c-Jun protein levels in T/C-28a2 chondrocytic cells. Cells were subjected to a shear stress level of 20 dyn/cm² for 24 h in either the absence or the presence of a JNK2 antisense oligonucleotide. A JNK2 sense oligonucleotide was used as a control. In static experiments, T/C28a2 cells were exposed to 0 dyn/cm² for 24 h. A, a representative Western hybridization experiment for COX-1 and COX-2. B, a similar experiment for JNK2, phosphorylated c-Jun (P-c-Jun), and total c-Jun. β -actin was probed as a loading control.

that inhibition of JNK2 or c-Jun, but not c-Fos, activity also abolishes EP2 and EP3a1 mRNA synthesis (Fig. 5, A and B), providing support to our hypothesis that these prostaglandin receptor subtypes represent downstream targets of COX-2 activity. The effects of antisense oligonucleotides on the shear regulation of the selected genes of this study were also validated using the cDNA microarray technology (data not shown).

Previous work has shown that COX-1, rather than COX-2, is involved in the regulation of the prostaglandin receptors in cervical carcinomas (18). Moreover, there is evidence, albeit contradictory (3, 13, 14), to suggest that fluid shear regulates COX-1 expression in human vascular cells. As a first step, we examined the effects of high shear stress (20 dyn/cm²) on COX-1 regulation in T/C-28a2 chondrocytic cells. The results indicate that COX-1 expression is not altered by fluid shear at either RNA or protein levels (Figs. 4A and 5, A and B). It is worth noting that inhibiting JNK2, c-Jun, or c-Fos transcripts does not affect COX-1 expression in the sheared *versus* static specimens either (Fig. 5, A and B). An RPA analysis of T/C-28a2 chondrocytic cells that were subjected to shear (20 dyn/cm² for 24 h) in the presence or absence of the specific COX-2 inhibitor NS-398 revealed that blockade of COX-2 activity abolished EP2 and EP3a1 mRNA up-regulation (Fig. 5, A and B). These observations were also confirmed using cDNA microarrays (data not shown). Taken together, these data indicate that shear-induced COX-2 activity, but not that of COX-1, regulates EP2 and EP3a1 mRNA synthesis. This observation is in clear contrast to previous work showing that COX-2 inactivation up-regulates prostaglandin EP3 and EP4 receptor expression in a murine kidney cell line (19).

Exposure of cartilage to abnormal mechanical loading may lead to cellular and biochemical changes that are associated



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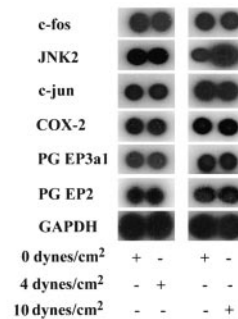


FIG. 5. A, effects of JNK2, c-Jun, and c-Fos antisense oligonucleotide and NS-398 (a specific COX-2 inhibitor) on shear-induced mRNA expression of c-Fos, JNK2, c-Jun, COX-2, COX-1, and prostaglandin receptors EP3a1 and EP2. Representative RPA gels show bands on each gene for static (control) and shear stress (20 dyn/cm², 24 h) conditions in the absence or presence of JNK2, c-Jun, or c-Fos antisense oligonucleotide (400 nM) or NS-398 (30 μ M). B, shear/static densitometry ratios. RPA densitometry ratios (normalized to GAPDH, which ranged from 0.97 \pm 0.01 to 1.01 \pm 0.01 for all treatments) are shown for seven genes, assessed after exposure of T/C28a2 chondrocytic cells to shear for 24 h in the absence or presence of antisense oligonucleotides or NS398. Values are mean \pm S.E. of three independent experiments. C, the effects of shear stress magnitude on mRNA expression of c-Fos, JNK2, c-Jun, COX-2, and prostaglandin receptors EP3a1 and EP2 for 24 h. Static controls were exposed to a magnitude of 0 dyn/cm² and paralleled the shear duration.

with cartilage degradation and the progression of arthritis. Nonsteroidal anti-inflammatory drugs represent an effective therapy for treating arthritic diseases, and elicit their responses by interfering with COX activity. We therefore wished to determine whether exposure of T/C-28a2 chondrocytic cells to lower levels of shear would induce COX-2 expression. The results indicate that application of a wall shear stress of 4 dyn/cm² to T/C-28a2 chondrocytic cells for a duration of 24 h did not significantly affect the gene transcript levels of the subset of genes examined in this work between the static and sheared samples (Fig. 5C). However, by increasing fluid shear to 10 dyn/cm², a significant up-regulation (4.7-fold) of the JNK2 transcript was detected by an RPA analysis, whereas the transcript levels of the other genes in the subset remained relatively unchanged (Fig. 5C). These findings underscore the effect of shear stress magnitude on gene regulation in

chondrocytic cells, and provide further support to the concept that JNK2 activation is the first step in the proposed cascade of events and is necessary for the regulation of COX-2 activity as well as downstream targets.

DISCUSSION

By coupling cDNA microarray technology with bioinformatics tools, we proposed a novel signaling pathway regulating COX-2 expression in chondrocytic cells exposed to mechanical stimulation. The conservation of the clustering patterns using two distinct algorithms provided evidence for the likely existence of the proposed pathway, which was validated using traditional molecular biology techniques. In particular, we demonstrate that fluid shear activates the signaling molecule JNK2, which then triggers the phosphorylation of the transcription factor c-Jun. These signaling events directly regulate COX-2 expression at both the mRNA and protein levels. Moreover, shear-induced COX-2 activity, but not COX-1, ultimately stimulates the synthesis of the prostaglandin receptor subtypes EP2 and EP3a1 in chondrocytic cells.

Previous studies on endothelial cells have shown that low levels of fluid shear (1–4 dyn/cm²) elicit COX-2 expression (12, 14). This finding is in clear contrast to the observations made in our study, in which induction of COX-2 was not detected after chondrocytic cell exposure to a shear stress level lower than 20 dyn/cm². The enhanced mechanosensitivity of human umbilical vein endothelial cells compared with chondrocytic cells may be related to the physiological shear environment (1–4 dyn/cm²) that these cells encounter *in vivo*. In contrast, chondrocytes reside in cartilage, which functions to absorb mechanical loading that arises during daily activities. Because abnormal mechanical loading of cartilage may be detrimental to the tissue, expression of proinflammatory genes such as 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.

Analysis of the human COX-2 gene has revealed the presence of regulatory sites, such as a TATA box, a CCAAT/enhancer-binding protein motif, two AP-2 sites, 3 SP-1 sites, two nuclear factor- κ B sites, a CRE motif, and an Ets-1 site (9). Previous studies have demonstrated the critical involvement of the CRE binding site in the shear-induced transcription of COX-2 in MC3T3-E1 osteoblastic cells (15) and endothelial cells (14). Nevertheless, the transcription factors and signaling intermediates regulating COX-2 induction have not previously been determined. In our study, we provide solid evidence that c-Jun regulates COX-2 transcription in T/C-28a2 chondrocytic cells stimulated with fluid shear. If CRE is indeed involved in the regulation of COX-2 in T/C-28a2 chondrocytic cells subjected to fluid shear, it is likely that c-Jun forms heterodimers with either members of the ATF family or c-Fos, which can bind to CRE sequences (34). However, microarray clustering analysis reveals that c-Fos is not found in the same subtree structure with COX-2 and c-Jun, suggesting that COX-2 regulation may be independent of c-Fos signaling. This is further substantiated by our functional knockout experiments using a c-Fos antisense oligonucleotide demonstrating that c-Fos inhibition does not affect COX-2 expression. This finding is in marked contrast to results obtained with chondrocytes stimulated with okadaic acid wherein c-Fos, JunB, and possibly c-Jun are involved in upstream regulatory binding (9). The aforementioned difference, as well as the absence of junB and junD regulation in response to shear (data not shown) as opposed to okadaic acid

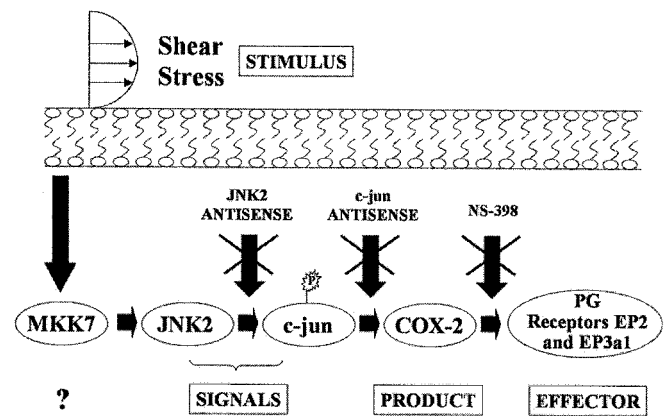


FIG. 6. Proposed cascade of signaling events that results from stimulation of T/C28a2 chondrocytic cells with fluid shear. Upon activation by high levels of shear stress, JNK2, possibly induced by MKK7, phosphorylates c-Jun, which in turn triggers the transcription of COX-2. Products of COX-2 activity, such as PGE₂, ultimately up-regulate the synthesis of PG receptor EP3a1 and E2 mRNA.

stimulation, support the notion that discrete signaling pathways are implicated in COX-2 regulation that are dependent on the stimulus imposed on the cell.

Using an antisense oligonucleotide directed against JNK2, we demonstrated its critical involvement in the biochemical pathway regulating shear-induced COX-2 expression in T/C-28a2 chondrocytic cells. More specifically, we showed that fluid shear activates JNK2, which in turn phosphorylates the transcription factor c-Jun. It is now established that JNK activation occurs via either mitogen-activated protein kinase kinase 7 (MKK7) or MKK4 phosphorylation of JNK Thr and Tyr (35). Although MKK4 can activate either the JNK or p38 pathways, MKK7 has been shown to function as a specific activator of JNK (36). Microarray analysis reveals an induction of MKK7, but not MKK4, in T/C-28a2 chondrocytic cells subjected to a shear stress level of 20 dyn/cm², with ratios of 3.40 and 8.33 after 1.5 and 24 h of stimulation, respectively. This pattern of MKK7 gene regulation parallels the expression of the JNK2, as evidenced by clustering algorithms (supplemental Fig. 1, *a* and *b*), thereby suggesting that MKK7 may be upstream of JNK2 activation (Fig. 6).

Noninvasive, pharmaceutical-based therapies for the treatment of arthritic diseases involve the use of nonsteroidal anti-inflammatory drugs, which elicit their effects by inhibiting COX activity and blocking the downstream production of prostanooids, including PGE₂. Although some *in vitro* studies have suggested possible anabolic effects associated with low concentrations of PGE₂ (37, 38), several lines of evidence indicate that PGE₂ is involved in cartilage erosion and inflammation associated with rheumatoid arthritis (17). Moreover, PGE₂ production in osteoarthritic cartilage has been found to be significantly elevated compared with that in normal tissue (39). It has been suggested that PGE₂ derived from COX-2 modulates the degradation of the cartilage proteoglycans in human osteoarthritic tissue stimulated with the pro-inflammatory cytokine interleukin-1 β (40). Although it is known that PGE₂ exerts its biological effects via its four prostaglandin receptors, very little is known about their contribution to the pathogenesis of arthritis. It was recently shown that mice lacking the EP4 receptor displayed a resistance to the development of experimentally induced arthritis (17). In our studies, we provide clear evidence that shear-induced COX-2 activity, but not COX-1, stimulates the synthesis of prostaglandin receptor subtypes EP2 and EP3a1, whereas no changes were observed for EP1 and EP4 as evidenced by microarray analysis (data not shown). The poten-

tial significance of EP2 and EP3a1 receptor subtypes in other models of arthritis (17) deserves further exploration.

In conclusion, using cDNA microarrays coupled with clustering algorithms followed by directed analysis of our candidate pathway, we elucidated a signaling mechanism regulating COX-2 expression in T/C-28a2 chondrocytic cells stimulated with fluid shear as well as downstream targets of COX-2 activity (Fig. 6). To our knowledge, this is the first application of cDNA microarray technology in conjunction with bioinformatics tools to generate a specific hypothesis about a candidate biochemical pathway in mammalian cells followed by its systematic analysis. This approach can further be exploited to propose novel regulatory networks in biological systems.

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