Shear-induced Interleukin-6 Synthesis in Chondrocytes

ROLES OF E PROSTANOID (EP) 2 AND EP3 IN cAMP/PROTEIN KINASE A- AND PI3-K/Akt-DEPENDENT NF-κB ACTIVATION

Pu Wang, Fei Zhu, Norman H. Lee, and Konstantinos Konstantopoulos

From the 4Department of Chemical and Biomolecular Engineering, 5Johns Hopkins Physical Science in Oncology Center, and 6Institute for NanoBioTechnology, The Johns Hopkins University, Baltimore, Maryland 21218 and the 7Department of Pharmacology and Physiology, The George Washington University Medical Center, Washington, D. C. 20037

Mechanical overloading of cartilage producing hydrostatic stress, tensile strain, and fluid flow can adversely affect chondrocyte function and precipitate osteoarthritis (OA). Application of high fluid shear stress to chondrocytes recapitulates the earmarks of OA, as evidenced by the release of pro-inflammatory mediators, matrix degradation, and chondrocyte apoptosis. Elevated levels of cyclooxygenase-2 (COX-2), prostaglandin (PG) E2, and interleukin (IL)-6 have been reported in OA cartilage in vivo, and in shear-activated chondrocytes in vitro. Although PGE2 positively regulates IL-6 synthesis in chondrocytes, the underlying signaling pathway of shear-induced IL-6 expression remains unknown. Using the human T/C-28a2 chondrocyte cell line as a model system, we demonstrate that COX-2-derived PGE2 signals via up-regulation of E prostanoid (EP) 2 and down-regulation of EP3 receptors to raise intracellular cAMP, and activate protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3-K)/Akt pathways. PKA and PI3-K/Akt transactivate the NF-κB p65 subunit via phosphorylation at Ser-276 and Ser-536, respectively. Binding of p65 to the IL-6 promoter elicits IL-6 synthesis in sheared chondrocytes. Selective knockdown of EP2 or ectopic expression of EP3 blocks PKA- and PI3-K/Akt-dependent p65 activation and markedly diminishes shear-induced IL-6 expression. Similar inhibitory effects on IL-6 synthesis were observed by inhibiting PKA, PI3-K, or NF-κB using pharmacological and/or genetic interventions. Reconstructing the signaling network regulating shear-induced IL-6 expression in chondrocytes may provide insights for developing therapeutic strategies for arthritic disorders and for culturing artificial cartilage in bioreactors.

Excessive chronic or repetitive mechanical loading of articular cartilage producing hydrostatic stress, tensile strain, and fluid flow (1) leads to irreversible cartilage erosion and osteoarthritic (OA) disease (2). Numerous in vitro studies support the concept that low fluid shear (<10 dyn/cm²) is chondroprotective (3), whereas high shear stress (>10 dyn/cm²) elicits the release of pro-inflammatory cytokines such as interleukin-6 (IL-6) (4), and mediates matrix degradation (3) and chondrocyte cell death (5, 6). Although OA is classified as a non-inflammatory joint disease, prostaglandins and cytokines are believed to play a role in the pathogenesis and progression of the disease. Prior work has shown that OA cartilage spontaneously releases prostaglandin (PG)E2 at 50-fold higher levels than in normal cartilage (7). The superinduction of PGE2 is mediated by cyclooxygenase-2 (COX-2) protein, whose levels are markedly up-regulated in OA-affected cartilage (7). The clinical correlates of OA are joint pain, dysfunction, and restricted motion. IL-6 has been implicated in pain signaling (8). Moreover, IL-6 mRNA expression occurs in human chondrocytes from OA but not normal cartilage (4). Catabolic and pro-inflammatory mediators such as PGE2 and IL-6 alter matrix homeostasis and participate in the destruction of articular cartilage, thereby contributing to OA.

Accumulating evidence suggests that IL-6 synthesis is positively regulated by either endogenous COX-2-derived or exogenously added PGE2 in many different cells including macrophages (9), osteoblasts (10), synovial (11), and gingival (12) fibroblasts. PGE2 exerts its biological functions via binding to four distinct transmembrane G-protein-coupled receptors (GPCRs) termed E prostanoid (EP) 1, EP2, EP3, and EP4. Following PGE2 binding, the EP receptors activate distinct intracellular signaling pathways, which may account for the pleiotropic effects of this prostaglandin. EP1 couples to Gαq protein and raises intracellular calcium (13). EP2 and EP4 elevate intracellular cAMP levels by activating adenylate cyclase via stimulatory G (Gs) proteins (13). The major signaling pathway of the EP3 receptor is the inhibition of adenylate cyclase, and thus reduction of intracellular CAMP, via inhibitory G (Gi) proteins (13). Human chondrocytes primed with exogenous PGE2 synthesize IL-6 via an unknown signaling pathway (14).

Although high fluid shear (16 dyn/cm²) has been reported to induce IL-6 mRNA and protein expression in human chondrocytes (4), the underlying mechanism of this process has yet to be elucidated. Because OA is often a consequence
of excessive mechanical forces (2) and that given application of high fluid shear to chondrocytes recapitulates the ear- 
marks of OA (3, 5, 6), we here delineate the signaling path-
way of IL-6 induction in shear-activated human T/C-28a2 
chondrocytes. Specifically, we demonstrate that COX-2-de-
pendent PGE2 signals via EP2 and EP3 receptors to regulate 
intracellular cAMP levels in chondrocytes, which in turn 
stimulate protein kinase A (PKA) and phosphatidylinositol
3-kinase (PI3-K)/Akt pathways. PKA and PI3-K/Akt trans-
activate the NF-κB p65 subunit via phosphorylation at Ser-
276 and Ser-536, respectively, which in turn 
binds to the p65 subunit via phosphorylation at Ser-
276, p-p65 (Ser-536), CREB, and p-CREB (Ser-133) were
modified Eagle’s medium supplemented with 10% fetal 
bovine serum (5, 16–18). Before shear exposure, cells were 
incubated for 18 h in serum-free medium supplemented with 
1% Nutridoma-SP (Roche), a low serum replacement that 
maintains chondrocyte phenotype and establishes quies-
cence in the monolayer (19, 20). Cells were then subjected to 
a shear stress level of 20 dyn/cm² for prescribed periods of 
time in medium containing 1% Nutridoma-SP, using a 
streamer gold flow device (Flexcell International, Hillsbor-
ough, NC). In select experiments, the pharmacological 
agents were added to the medium at the indicated concen-
trations just before the onset of shear exposure. It is well 
established that transmission of the shear stress signal 
throughout the cell involves a complex interplay between 
cytoskeletal and biochemical constituents, and results in 
changes in structure, metabolism, and gene expression (21).

**EXPERIMENTAL PROCEDURES**

**Reagents**—The COX-2 selective inhibitor NS398, the EP 
and DP receptor antagonist AH6809, the selective agonist 
for EP3 receptor sulprostone, PGE₂, forskolin, the PKA
inhibitor H89, and the NF-κB inhibitor 6-amino-4-[(4-
phenoxyphenylethylamino) quinazoline (QNZ) were obtained 
from Enzo Life Sciences International Inc (Plymouth Meet-
ing, PA). The EP3 and p65 cDNA plasmids were supplied 
from Origene Technologies (Rockville, MD) and subcloned 
to the pcMV6-XL vector. The IL-6 promoter reporter con-
structs pIL6-luc651 (-651/+1) and pIL6-luc651 ∆NF-κB (NF-
κB site mutation) were gifts from Dr. Eickelberg (15).
The PI3-K inhibitors, LY294002 and wortmannin, were from 
Sigma-Aldrich. p65 siRNA and antibodies specific for β-ac-
tin, COX-2, Akt, p-Akt (Ser-473), NF-κB p65, p-p65 (Ser-
276), p-p65 (Ser-536), CREB, and p-CREB (Ser-133) were
purchased from Cell Signaling Technology, Inc. (Danvers, MA). 
CREB1 and ATF4 siRNAs as well as monoclonal antibodies specific 
for p65 and IL-6 were obtained from Santa Cruz Biotechnology, 
Inc (Santa Cruz, CA). The cAMP
enzyme immunoassay kit and antibodies specific for EP2 and 
EP3 receptors were from Cayman Chemical. All reagents for qRT-
PCR and SDS-PAGE experiments 
were purchased from Bio-Rad Laboratories. Reagents for elec-
rophoretic mobility shift assays (EMSA) were obtained from 
Pierce Chemical Company. The Dual-Luciferase Reporter Assay 
kit was purchased from Promega (Madison, WI). All other reagents 
were from Invitrogen (Carlsbad, CA), unless otherwise specified. 

**Cell Culture and Shear Stress 
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**Exposure—**Human primary arti-
cular chondrocytes (Cell Applica-
tions) or T/C-28a2 chondrocytic 
cells were grown (37 °C in 5% CO₂) 
on glass slides in F12/Dulbecco’s 
modified Eagle’s medium supplemented with 10% fetal 
bovine serum (5, 16–18). Before shear exposure, cells were 
incubated for 18 h in serum-free medium supplemented with 
1% Nutridoma-SP (Roche), a low serum replacement that 
maintains chondrocyte phenotype and establishes quies-
cence in the monolayer (19, 20). Cells were then subjected to 
a shear stress level of 20 dyn/cm² for prescribed periods of 
time in medium containing 1% Nutridoma-SP, using a 
streamer gold flow device (Flexcell International, Hillsbor-
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agents were added to the medium at the indicated concen-
trations just before the onset of shear exposure. It is well 
established that transmission of the shear stress signal 
throughout the cell involves a complex interplay between 
cytoskeletal and biochemical constituents, and results in 
changes in structure, metabolism, and gene expression (21).

**Transient Transfection and Reporter Gene Assays**—For 
ectopic expression of EP3 receptor or p65, T/C-28a2 chon-
drocytes were transfected with 1.6 μg/slide of plasmid con-
taining the EP3 or p65 cDNA by using Lipojette 2000. In 
control experiments, cells were transfected with 1.6 
μg/slide of the empty vector pcMV6-XL (OriGene Technolo-
gies). In select experiments, T/C-28a2 cells were trans-
fected with 1.6 μg/slide of the IL-6 promoter reporter con-
struct pIL6-luc651 or pIL6-luc651 ∆NF-κB. In RNA 
interference assays, T/C-28a2 cells were transfected with 
100 nm siRNA oligonucleotide sequence specific for CREB1, 
ATF4, or p65. In control experiments, cells were transfected 
with 100 nm control siRNA. For EP2 shRNA experiments, 
T/C-28a2 chondrocytes were transfected with 1.6 μg/slide
of plasmid containing the EP2 shRNA or scramble shRNA control. The following oligonucleotide sequences were used to specifically target the EP2 mRNA (underlined, sense and antisense sequences; boldface italicized, loop with linker):

- **Top strand**, 5′-H11032-GATCCCCGATCCAGCTGCCTATTGATTCAAGAGAATCAATAGGCAGCTGGATCTTTTTC-3′/H11032;
- **Bottom strand**, 5′/H11032-TCGAGAAAAAGATCCAGCTGCCTATTGATTCTCTTGAAATCAATAGGCAGCTGGATCGG-3′/H11032.

Top and bottom strands were annealed, subcloned into the XhoI/BglII sites of pSuper, and the resulting construct sequence was verified. Transfected cells were allowed to recover for at least 12 h in growth medium and then incubated overnight in medium containing 1% Nutridoma-SP before their exposure to shear or static conditions. In promoter activity experiments, luciferase activities were measured by using the Dual-Luciferase Reporter Assay kit (Promega), as previously described (5, 17).

**Quantitative Real-Time PCR (qRT-PCR)**—qRT-PCR assays were performed on the iCycler iQ detection system (Bio-Rad) using total RNA, the iScript one-step RT-PCR kit with SYBR green (Bio-Rad) and primers. The GenBank accession numbers and forward (F-) and reverse (R-) primers are as follows:

- **Cox-2 (NM_000963)**, F-TGAGCATCTACGGTTTGCTG, R-AACTGCTCATCACCACCCATT;
- **EP1 (NM_000955)**, F-TGGGCCTCTGGTTGTGCTTA, R-TTCCGGCTCTCCACCTTCTTTG;
- **EP2 (NM_000956)**, F-CGTGCACCTACTTCGCTTTC, R-GAGGTCCCATTTTTCCTTTC;
- **EP3 (NM_198712)**, F-TTCTGCCCGCCCTCAACCA, R-AGGAGAGCCGGAAAAACGTCA;
- **EP4 (NM_000958)**, F-TGGCCGCAAAGCAGAAGGAGACG, R-GGACGGTGGCGAGAATGAGGAAGG;
- **IL-6 (NM_000600)**, F-ATGAACTCCTTCTCCACAAGCGC, R-GAAGAGCCCTCAGGCTGGACT;
- **CREB1 (NM_134442)**, F-CCAGGTATCTATGCCAGCAG, R-TCTGGTTCCGGAGAAAAGTC;
- **ATF4 (NM_182810)**, F-CATTCCTCAGATTCCAGCAAAGCAC, R-CTTCCTCAGTTCCCAAGCCC;
- **p65 (NM_001145138)**, F-CTGCAGTTTGATGATGAAGA, R-TAGGCGAGTTATAGCCTCAG;
- **GAPDH (NM_002046)**, F-CCACCCATGGCAAATTCCATGGCA, R-TCTAGACGGCAGGTCAGGTCACC.

GAPDH was used as internal control. Reaction mixtures were incubated at 50 °C for 15 min followed by 95 °C for 5 min, and then 35 PCR cycles were performed with the following temperature profile: 95 °C 15 s, 58 °C 30 s, 68 °C 1 min, 77 °C 20 s. Data were collected at the (77 °C 20 s) step to remove possible fluorescent contribution from dimer primers (22). Gene expression values were normalized to GAPDH.

**Western Blot Analysis**—T/C-28a2 cells, from sheared and matched static control specimens, were lysed in radioimmune precipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS) containing a mixture of proteinase inhibitors (Pierce). The protein content of the cell lysates was determined using bicinechonic acid (BCA) protein assay reagent (Pierce). Total cell lysates (4 μg) were subjected to SDS-PAGE, transferred to a...
membrane, and probed with a panel of specific antibodies. Each membrane was only probed using one antibody. β-Actin was used as loading control. All Western hybridizations were performed at least in triplicate using a different cell preparation each time.

Preparation of Cytosolic and Nuclear Extracts—Cytosolic and nuclear extracts were isolated using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) following the manufacturer’s instructions as previously described (17).

EMSA and Supershift Assay—A 5′-biotinylated oligonucleotide probe (5′-GGGATTTTCC-3′) was synthesized containing the NF-κB cis-element present on the IL-6 promoter. EMSAs were performed with a commercially available nonradioisotopic EMSA kit (LightShift Chemiluminescence EMSA kit; Pierce). Briefly, nuclear extracts (1–2 µg) were incubated in 10× binding buffer (supplemented with 50 ng of poly(dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, 5 mM MgCl₂, and 0.25 mg of bovine serum albumin), containing 20 fmol of biotinylated, double-stranded probe for NF-κB for 30 min on ice. 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 on ice with an anti-p65 antibody. The biotinylated oligonucleotide probe specific for NF-κB was then added to the reaction mixture and incubated for another 30 min on ice. To exclude the possibility of nonspecific binding, a 5′-biotinylated random probe (5′-TCATTTTGTC-3′) designed using a random sequence generator was used in shift and supershift assays (supplemental Fig. S1). The protein-DNA complexes were resolved on a native 6% polyacrylamide retardation gel in 0.5× Tris borate-EDTA running buffer at 10 mA for 1 h, transferred to a nylon membrane (Pierce), visualized using the LightShift Chemiluminescence kit (Pierce) and exposed to Kodak x-ray film (Pierce).

Measurement of Intracellular cAMP Concentration—cAMP levels were determined by cyclic AMP enzyme immunoassay kit following the manufacturer’s instructions (Cayman Chemical). The protein concentration of total cell lysate was used as loading control, and the results were expressed as pmol of cAMP/µg of total protein.

RESULTS

Shear Stress Induces IL-6 Expression in Human Chondrocytes via a COX-2-dependent Pathway—IL-6 expression has been detected in chondrocytes from OA but not normal cartilage (4). Prior work has shown that high fluid shear stress (16 dyn/cm²) induces IL-6 production in human articular chondrocytes in vitro (4). However, the signaling pathway of IL-6 induction in human chondrocytes in response to elevated levels of fluid shear remains unknown. In light of accumulating evidence suggesting that OA is often a consequence of abnormal mechanical forces (23), we here aimed to delineate the mechanism by which shear stress induces IL-6 expression in human chondrocytes. The human T/C-28a2 chondrocyte cell line was chosen as a model system, because T/C-28a2 cells have been shown to behave much like primary human chondrocytes when cultured under appropriate conditions (19, 20). In view of previous observations showing a positive correlation between endogenous COX-2-mediated PGE₂ production and IL-6 synthesis (24, 25), we first evaluated the effects of shear stress on COX-2 expression and formation of cAMP, because PGE₂ is a well-known activator of the cAMP signaling pathway. In accord with prior data (17), high shear stress (20 dyn/cm²) rapidly induces COX-2 mRNA (Fig. 1A) and protein (Fig. 1B) expression in human T/C-28a2 chondrocytes and cAMP production (Fig. 1C). IL-6 mRNA and protein up-regulation follows later, after 30–60 min of cell stimulation with fluid shear (Fig. 1, D and E). Treatment of T/C-28a2 chondrocytes
with the selective COX-2 inhibitor NS398 (30 μM) markedly suppresses both intracellular cAMP accumulation (Fig. 1C) and IL-6 mRNA expression (Fig. 1D), suggesting the potential involvement of cAMP in shear-induced IL-6 synthesis in human chondrocytes. To validate previously published observations suggesting that T/C-28a2 cells represent an appropriate model for studying chondrocyte function in vitro (19, 20), we examined the responses of human primary articular chondrocytes to shear stress (20 dyn/cm²). Our data revealing significant similarities between primary articular chondrocytes and T/C-28a2 cells in the induction of IL-6 mRNA synthesis in response to shear stress (Fig. 1D) reinforce the aforementioned notion.

Shear-induced IL-6 mRNA Synthesis Proceeds via an EP2-/EP3-dependent Mechanism—The effects of PGE2 are mediated via four different transmembrane GPCRs, namely EP1–4, which are involved in the activation of phospholipase C (EP1) and activation (EP2, EP4) or inhibition (EP3) of adenyl cyclase (13). In agreement with previously published data from primary human chondrocytes (26), T/C-28a2 cells express EP2, EP3 and very low levels of EP4 receptors (supplemental Fig. S2A). As a next step, we assessed the influence of shear stress on EP receptor expression. Application of high fluid shear up-regulates EP2 (Fig. 2, A and C) while it down-regulates EP3 (Fig. 2, B and D) receptor expression at both the mRNA and protein levels in T/C-28a2 chondrocytes. No significant changes are noted in the mRNA expression levels of EP1 and EP4 receptors in sheared relative to static control T/C-28a2 chondrocytes (supplemental Fig. S2B). The divergent effects of shear stress on EP2 and EP3 receptor expression are in line with the enhanced cAMP accumulation observed in shear-activated chondrocytes (Fig. 1C). Thus, we next investigated the effects of an EP2 receptor antago-
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Shear Stress Induces PI3-K and PKA Activation in Human Chondrocytes and Mediates IL-6 Synthesis via a COX-2/EP2/EP3-dependent Pathway—We next aimed to delineate the signaling mechanism of IL-6 up-regulation in sheared chondrocytes. In view of our observations showing that shear-induced cAMP formation precedes IL-6 mRNA expression in human chondrocytes (Fig. 1), we examined the potential contribution of downstream effectors of cAMP activity to this signaling cascade, which include PI3-K and PKA (29, 30). Exposure of T/C-28a2 chondrocytes to high shear stress increases the phosphorylation levels of Akt at Ser-473 and CREB at Ser-133 without affecting total Akt and CREB levels (Fig. 4). The shear-induced Akt and CREB phosphorylation are significantly suppressed by treating T/C-28a2 cells with the selective COX-2 inhibitor NS398 (Fig. 4A), the EP2 receptor antagonist AH6809 or the EP3 receptor agonist sulprostone (Fig. 4B). Along these lines, selective EP2 receptor knockdown or EP3 receptor overexpression (Fig. 4C) reverses shear-induced Akt and CREB phosphorylation to near background levels in T/C-28a2 chondrocytes.

To evaluate the involvement of PI3-K in the regulation of shear-induced IL-6 mRNA synthesis in human chondrocytes, T/C-28a2 cells were treated with the selective PI3-K inhibitors, LY294002 (30 μM) or wortmannin (10 μM). These pharmacological inhibitors abrogate the phosphorylation of Akt at Ser-473 without affecting total Akt levels (Fig. 5D), and markedly attenuate shear-induced IL-6 mRNA synthesis (Fig. 5A and B). Furthermore, treatment of T/C-28a2 chondrocytes with the PKA inhibitor H89 (10 μM) drastically diminishes the induction of IL-6 mRNA expression in response to high fluid shear stimulation (Fig. 5C). This pharmacological intervention is also effective in suppressing CREB phosphorylation at Ser-133, while it appears to increase Akt phosphorylation at Ser-473 (Fig. 5D), which is in line with a potential crosstalk between PI3-K and PKA (29). Cumulatively, our data suggest that shear stress induces IL-6 mRNA synthesis in human chondrocytes via PKA- and PI3-K/Akt-dependent pathways, which are in turn regulated by a COX-2/EP2/EP3-dependent mechanism.

NF-κB p65 Subunit, but Not CREB, Is Involved in Shear-induced IL-6 mRNA Synthesis—The promoter of the IL-6 gene contains several cis-elements including a cAMP response element (CRE) motif, NF-κB, and AP-1 sites, which have been...
implicated in the induction of IL-6 in different cell types (9, 31). In light of our observations showing that fluid shear induces CREB phosphorylation (Fig. 4), we assessed the potential contributions of CREB1 and CREB2 (also called ATF4) to IL-6 induction using T/C-28a2 cells transfected with either an siRNA oligonucleotide sequence specific for CREB1 or ATF4, or control siRNA. The efficacy of CREB1 and ATF4 knockdown is documented at the transcript level, whereas the control siRNA fails to alter CREB1 and ATF4 mRNA expression levels (Fig. 6, B and C). Simultaneous knockdown of CREB1 and ATF4 does not impair the extent of shear-induced IL-6 mRNA synthesis (Fig. 6A), suggesting the absence of their functional role in this process.

The potential involvement of NF-κB in the induction of IL-6 in shear-activated chondrocytes was first disclosed by gel and supershift assays. Incubation of nuclear extracts from sheared versus untreated T/C-28a2 chondrocytes with the biotinylated NF-κB probe leads to the formation of the NF-κB specific DNA-protein complex (Fig. 7A). Furthermore, incubating nuclear extracts from shear-activated T/C-28a2 cells with an anti-p65 antibody prior to the addition of the biotinylated NF-κB probe results in a marked supershift of the complex (Fig. 7B). Both the formation of the NF-κB-specific DNA-protein complex and the supershift are inhibited by treating T/C-28a2 chondrocytes with the selective COX-2 inhibitor NS398 (Fig. 7, A and B). Similarly, EP2 receptor knockdown or EP3 receptor overexpression are effective in antagonizing the NF-κB-specific gel shift and supershift (Fig. 7, C and D). Moreover, the PI3-K inhibitors LY294002 or wortmannin (Fig. 8, A and B) or the PKA inhibitor H89 (Fig. 8, B and D) exert similar inhibitory effects.

Application of fluid shear to T/C-28a2 chondrocytes induces p65 phosphorylation at both Ser-536 and Ser-276 without affecting total p65 protein levels (Fig. 4). Treatment of cells with the selective COX-2 inhibitor NS398 (30 μM) diminishes shear-induced p65 phosphorylation at both sites down to basal levels (Fig. 4A). Akin inhibitory effects are noted in sheared T/C-28a2 cells pretreated with either the EP2 receptor antagonist AH6809 or the EP3 receptor agonist sulprostone (Fig. 4B). Similarly, EP2 receptor knockdown or EP3 receptor overexpression inhibits the shear-induced phosphorylation of p65 at both sites. The PI3-K inhibitors, LY294002 (30 μM) or wortmannin (10 μM), nearly abrogate p65 phosphorylation at Ser-536 while leaving intact the phosphorylation at Ser-276 in shear-activated T/C-28a2 chondrocytes (Fig. 5D). It is noteworthy that the PKA inhibitor H89 (10 μM) has the reverse effects on p65 phosphorylation (Fig. 5D).

To demonstrate the functional contribution of NF-κB to the shear-induced IL-6 mRNA synthesis in T/C-28a2 chondrocytes, cells were incubated with the NF-κB inhibitor QNZ (10 μM). This treatment significantly diminishes the induction of IL-6 mRNA in shear-activated T/C-28a2 chondrocytes.
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FIGURE 8. Fluid shear differentially modulates the expression of EP2 and EP3 receptors, which in turn activate PKA and PI3-K signaling pathways that mediate NF-κB activation in human chondrocytes. T/C-28a2 cells were subjected to shear stress (20 dyn/cm²) or static conditions (0 dyn/cm²) for 1 or 2 h in the absence or presence of the PI3-K inhibitors (LY294002 (30 μM) or wortmannin (10 μM)) or the PKA inhibitor H89 (10 μM). Nuclear extracts were prepared for the determination of NF-κB-specific DNA-protein complex formation by EMSA (A, B). Supershift (C, D) assays using an anti-p65 Ab were carried out as outlined under “Experimental Procedures.” Results of a competition experiment using 50-fold unlabeled NF-κB oligonucleotide (cold probe) are shown. These gels are representative of three independent experiments, all revealing similar results.

(Fig. 9A), and markedly attenuates the NF-κB-specific gel shift and supershift (Fig. 9, B and C). To confirm the key role of the NF-κB p65 subunit in the regulation of shear-induced IL-6 mRNA expression, experiments were performed using human T/C-28a2 chondrocytes transfected with either an siRNA oligonucleotide sequence specific for p65. This genetic intervention effectively knocks down p65 mRNA expression in sheared T/C-28a2 chondrocytes relative to cells transfected with a scramble siRNA control (Fig. 10A), and abrogates shear-induced IL-6 mRNA synthesis (Fig. 10B). Moreover, p65 knockdown inhibits the formation of the NF-κB specific DNA-protein complex and its supershift (Fig. 10, C and D).

We next examined the effects of shear stress on IL-6 promoter activity in T/C-28a2 chondrocytes transiently transfected with a construct encompassing the 5′-flanking region of the human IL-6 gene from −651 to +1 bp (−651/+1) cloned into a promoterless luciferase expression vector (15). As shown in Fig. 10E, application of shear stress (20 dyn/cm² for 60 min) to T/C-28a2 chondrocytes increases the IL-6 promoter activity relative to static controls. Introduction of a mutation into the NF-κB binding site (−72/−63) nearly abrogates the shear-induced luciferase activity relative to the wild-type reporter (Fig. 10E), thereby providing additional evidence for the key role of NF-κB in the regulation of shear-induced IL-6 expression in human T/C-28a2 chondrocytes.

Given the critical role of the NF-κB p65 subunit in induction of IL-6 in shear-activated chondrocytes, we next investigated whether ectopic expression of p65 is sufficient to induce IL-6 mRNA synthesis in statically incubated T/C-28a2 cells. Transfection of T/C-28a2 cells with a plasmid containing the p65 cDNA markedly increases the p65 mRNA (Fig. 11A) and protein levels (Fig. 11B) relative to cells transfected with a vector control. Ectopic expression of p65 also triggers the translocation of p65 from the cytosol to the nucleus in human T/C-28a2 chondrocytes. Moreover, this genetic intervention induces the IL-6 mRNA synthesis (Fig. 11A), the formation of the NF-κB-specific DNA-protein complex and its supershift (Fig. 11C) as well as promoter activity (Fig. 11D).

Taken together, these data reveal that high shear stress induces PI3-K and PKA activation in human chondrocytes via COX-2-dependent cAMP production mediated via EP2 receptor up-regulation and EP3 receptor down-regulation (Fig. 12). PI3-K and PKA in turn transactivate the NF-κB p65 subunit via phosphorylation at Ser-536 and Ser-276, respectively (Fig. 12). Binding of p65 to the IL-6 promoter elicits shear-induced IL-6 mRNA synthesis in sheared T/C-28a2 chondrocytes (Fig. 12).

DISCUSSION

Excessive mechanical loading can directly damage the articular cartilage, adversely affect chondrocyte function, and precipitate OA (2). IL-6 expression (4) as well as superinduction of COX-2 accompanied by markedly increased levels of PGE2 release (7) have been detected in OA-affected cartilage and in shear-activated human chondrocytes in vitro (4, 5). In view of the involvement of IL-6 in pain signaling associated with arthritic disorders (8) and the role of mechanical forces in the pathogenesis and progression of OA, we here delineated the signaling pathway of its induction in mechanically stimulated...
human T/C-28a2 chondrocytes. We demonstrate that shear-induced COX-2-derived PGE$_2$ signals via the EP2 and EP3 receptors to mediate intracellular cAMP accumulation, which in turn regulates PKA- and PI3-K/Akt-dependent NF-$\kappa$B activation and IL-6 mRNA and protein expression in human T/C-28a2 chondrocytes (Fig. 12).

PGE$_2$ exerts its biological actions by binding to a group of four GPCRs. Li et al. (14) reported that EP2 is the major mediator of PGE$_2$-induced protoclycan accumulation in articular cartilage-degrading enzymes, such as matrix metalloproteinase-13, in human OA chondrocytes occurs via an EP4-dependent/EP2-independent signaling pathway. In contrast, Li et al. (14) reported that EP2 is the major mediator of PGE$_2$-induced suppression of proteoglycan accumulation in articular chondrocytes, which is not accompanied by any significant modulation of MMP activity. Their data (14) are in accord with our results, which suggest that inhibition of the EP receptor signaling may be beneficial to antagonize the catabolic activity of PGE$_2$ in articular cartilage and the IL-6-dependent pain symptoms associated with OA in human joints. In agreement with these observations, Chen et al. (9) recently reported that an EP2 agonist induces IL-6 production in RAW 264.7 macrophages.

The dominant role of EP2 has also been documented in the regulation of tumor necrosis factor (TNF)-$\alpha$-induced IL-6 production in human rheumatoid arthritis activated synovial fibroblasts (RASFs) (33). Although blockade of EP2 activity reduces TNF-$\alpha$-mediated IL-6 synthesis in human RASFs (33), an EP2 agonist attenuates IL-6 production in IL-1-activated rat synovial cells (34). However, all the aforementioned previously-published results regarding the involvement of distinct EP receptors in the regulation of IL-6 synthesis and matrix degradation need to be interpreted with caution, since they were acquired using rather non-selective pharmacological agents. To validate our findings, we also used genetic interventions to knock down IL-6 expression. Our data further reveal that the EP2/EP3-dependent up-regulation of cell cAMP promotes PKA- and PI3-K/Akt activation, as evidenced by the increased phosphorylated levels of CREB and Akt, respectively. Prior work has suggested that EP4 rather than EP2 stimulation preferentially results in the activation of the PI3-K-dependent pathway (30). However, the results of this study disclose that selective EP2 knockdown or EP3 overexpression dramatically attenuates the phosphorylation levels of Akt in sheared chondrocytes. Our data along with previously published results (30) suggest that all EP receptors that are capable of effectively modulating intracellular cAMP levels have the capability to regulate the PI3-K pathway. Their specific contribution to this process may be related to the modulation of their relative expression levels and activity by the application of an external signal.

Numerous studies suggest that PGE$_2$ exerts catabolic effects in articular cartilage (14, 27, 32), as evidenced by the decreased aggrecan synthesis and total proteoglycan accumulation. Attur et al. (27) concluded that the PGE$_2$-mediated proteoglycan degradation and up-regulation of cartilage-degrading enzymes, such as matrix metalloproteinase-13, in human OA chondrocytes occurs via an EP4-dependent/EP2-independent signaling pathway. In contrast, Li et al. (14) reported that EP2 is the major mediator of PGE$_2$-induced suppression of proteoglycan accumulation in articular chondrocytes, which is not accompanied by any significant modulation of MMP activity. Their data (14) are in accord with our results, which suggest that inhibition of the EP receptor signaling may be beneficial to antagonize the catabolic activity of PGE$_2$ in articular cartilage and the IL-6-dependent pain symptoms associated with OA in human joints. In agreement with these observations, Chen et al. (9) recently reported that an EP2 agonist induces IL-6 production in RAW 264.7 macrophages.
the EP2 receptor via RNAi or overexpress the EP3 receptor. These interventions interfere with intracellular cAMP accumulation, and PKA-/Akt-dependent NF-κB activation and IL-6 synthesis in sheared chondrocytes.

The promoter of the human IL-6 gene contains several cis-elements that can bind diverse trans-acting factors such as CREB and NF-κB, which have been implicated in the induction of IL-6 in other cell types (9, 31). Even though fluid shear up-regulates CREB phosphorylation in T/C-28a2 chondrocytes, knockdown of both CREB1 and CREB2 (ATF4) fails to interfere with the induction of IL-6 synthesis, suggesting the lack of their functional contribution to this process. Shear activation of human T/C-28a2 chondrocytes enhances the formation of NF-κB-specific DNA-protein complex. Furthermore, incubation of nuclear extracts from shear-stimulated T/C-28a2 chondrocytes with an anti-p65 antibody results in a marked supershift of the complex. Use of the selective COX-2 inhibitor significantly reduces the shear-induced NF-κB activation in both gel shift and supershift assays. EP2 receptor knockdown or EP3 receptor overexpression leads to similar inhibitory effects. Akin results are obtained by inhibiting the downstream targets of cAMP, PKA, and PI3-K. Through the use of RNAi, we demonstrated the functional role of the NF-κB p65 subunit in shear-induced IL-6 mRNA synthesis in human T/C-28a2 chondrocytes. In agreement with our previously published results (17), p65 knockdown did not affect the extent of shear-induced COX-2 expression in chondrocytes (data not shown), thereby suggesting that COX-2 is upstream of NF-κB and eliminating the possibility of a feedback mechanism from NF-κB to COX-2. The key role of NF-κB in shear-induced IL-6 transcriptional activity is further demonstrated by the use of wild-type and NF-κB mutated IL-6 promoter-reporter constructs. Interestingly, ectopic expression of p65 in human chondrocytes stimulates IL-6 mRNA synthesis.

The NF-κB p65 subunit is phosphorylated at both Ser-276 and Ser-576 in shear-activated human chondrocytes. Similar observations were made for T/C-28a2 chondrocytes stimulated with exogenously added PGE2 or forskolin (supplemental Fig. S3). The selective COX-2 inhibitor NS398 as well as EP2 knockdown or EP3 overexpression inhibit shear-induced p65 phosphorylation at both sites, suggesting that p65 phosphorylation occurs downstream of COX-2 generated PGE2. Inhibition of PKA by H89 and PI3-K by wortmannin and LY294002 block p65 phosphorylation at Ser-276 and Ser-576, respectively. Each pharmacological inhibitor alone diminishes shear-induced IL-6 synthesis, thereby suggesting that p65 phosphorylation at both Ser-276 and Ser-576 is essential for maximal induction of IL-6 in shear-activated human chondrocytes.

A wide array of pro-inflammatory mediators, including IL-6, IL-1β, and TNF-α, are up-regulated in OA cartilage relative to healthy controls (35). IL-1β and TNF-α are thought to play a
pivotal role in cartilage degradation in OA patients, by stimulating chondrocytes and synoviocytes to produce matrix proteases and prostaglandins, among others (35). Accumulating evidence suggests that nearly all pro-inflammatory mediators involved in pathogenesis and progression of OA and rheumatoid arthritis are modulated by NF-κB (36). Thus, NF-κB is an important therapeutic target for arthritic diseases. Indeed, inhibition of NF-κB represses the progression of the early experimental OA (37) and joint swelling in mice with collagen-induced arthritis (38). Our data showing the key role of NF-κB in the induction of IL-6 in shear-activated chondrocytes further underscore its involvement in pain signaling associated with OA.

In conclusion, we demonstrate that high fluid shear regulates IL-6 synthesis in human chondrocytes via COX-2-derived PGE2 signaling that proceeds through an EP2-/EP3-dependent mechanism to upregulate cAMP and mediate PKA- and PI3-K-dependent p65 binding to the IL-6 promoter. Reconstructing the signaling pathway regulating the catabolic responses of chondrocytes induced by excessive shear stress may identify additional potential therapeutic targets for controlling OA pathogenesis and/or progression and may be useful in the design of bioreactors for cartilage tissue engineering applications.

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