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Shear- and Compression-induced Chondrocyte Transcription Requires MAPK Activation in Cartilage Explants*

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Chondrocytes regulate the composition of cartilage extracellular matrix in response to mechanical signals, but the intracellular pathways involved in mechanotransduction are still being defined. Mitogen-activated protein kinase (MAPK) pathways are activated by static and dynamic compression of cartilage, which simultaneously induce intratissue fluid flow, pressure gradients, cell, and matrix deformation. First, to determine whether cell and matrix deformation alone could induce MAPK activation, we applied dynamic shear to bovine cartilage explants. Using Western blotting, we measured ERK1/2 and p38 activation at multiple time points over 24 h. Distinct activation time courses were observed for different MAPKs: a sustained 50% increase for ERK1/2 and a delayed increase in p38 of 180%. We then investigated the role of MAPK activation in mechano-induced chondrocyte gene expression. Cartilage explants were preincubated with inhibitors of ERK1/2 and p38 activation before application of 1–24 h of three distinct mechanical stimuli relevant to *in vivo* loading (50% static compression, 3% dynamic compression at 0.1 Hz, or 3% dynamic shear at 0.1 Hz). mRNA levels of selected genes involved in matrix homeostasis were measured using real-time PCR and analyzed by k-means clustering to characterize the time- and load-dependent effects of the inhibitors. Most genes examined required ERK1/2 and p38 activation to be regulated by these loading regimens, including matrix proteins aggrecan and type II collagen, matrix metalloproteinases MMP13, and ADAMTS5, and transcription factors downstream of the MAPK pathway, c-Fos, and c-Jun. Thus, we demonstrated that the MAPK pathway is a central conduit for transducing mechanical forces into biological responses in cartilage.

In vitro models of chondrocyte mechanobiology relevant to physiological loading of cartilage *in vivo* have shown that biosynthesis and gene transcription of the major matrix proteins in cartilage are regulated by distinct tissue deformations (reviewed in Ref. 1). Application of static compression to intact cartilage explants is inhibitory to proteoglycan and type II collagen production and transcription (2–7), whereas dynamic loading regimens, including dynamic compression and dynamic shear deformation, generally enhance matrix protein biosynthesis and transcription (4, 8–13). Differences in intracellular signaling between normal and osteoarthritic chondrocytes following mechanical loading (14–16) have led to speculation that altered mechanotransduction may be a cause or result of the development of osteoarthritis following injury. Therefore, understanding the pathway(s) by which chondrocytes transduce mechanical forces into biological responses is of great importance to the treatment of osteoarthritis and to cartilage tissue engineering. The mitogen-activated protein kinase (MAPK)[5] pathway has been implicated in chondrocyte mechanotransduction responses. In cartilage explants, static compression can induce the phosphorylation of extracellular signal-regulated kinases (ERK1/2) and p38 (17), and dynamic compression can induce ERK activation (18). In isolated chondrocytes, fluid shear activates ERK1/2 (19).

ERK1/2, p38, and JNK are the central components of the MAPK cascade that coordinate incoming signals generated by a variety of extracellular and intracellular mediators in many cell types. The phosphorylation and activation of MAPKs transmits the signal down the cascade, resulting in the activation of many downstream regulatory proteins such as other protein kinases, transcription factors, cytoskeletal proteins, and other enzymes (for review (20)). In chondrocytes, the MAPKs are known to play an important role in response to inflammatory cytokines (21, 22). For example, interleukin-1β (IL-1β) and tumor necrosis factor α regulate the transcription and synthesis of a number of matrix metalloproteinases (MMPs) (23–25) through the transient activation of ERK1/2, p38, and JNK in chondrocytes

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[5] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; IL-1, interleukin-1; MMP, matrix metalloproteinase; TBST, tris-buffered saline Tween-20; TIMP, tissue inhibitor of matrix protease; ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; MAPKAPK2, mitogen-activated protein kinase activated protein kinase 2; ECM, extracellular matrix; COX2, cyclooxygenase 2; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

(21, 26–32). Interestingly, regulatory binding sites for transcription factors that are downstream targets of the MAPKs, such as activating protein-1, are present in the promoter regions of MMP1, MMP3, and MMP13 (22, 23, 33). There is some evidence that the MAPK pathway may also mediate effects of anabolic growth factors in cartilage (such as insulin-like growth factor-1, fibroblast growth factor, transforming growth factor-β) (17, 34–37).

We have previously shown that mechanical loads applied to intact cartilage explants, which imitate different aspects of *in vivo* loading, regulate a number of genes involved in cartilage homeostasis, including matrix proteins, matrix proteases, TIMPs, transcription factors, and signaling molecules (6, 8). Using clustering techniques we demonstrated that genes responded in functionally related groups and in a specific time- and load-dependent manner, with a number of genes requiring calcium-activated pathways. Here we investigate the role of MAPK signaling in mechano-regulation of gene expression by utilizing real-time PCR, gene clustering techniques (38–40), and molecular inhibitors of ERK, p38, and protein synthesis. First, we confirm that ramp-and-hold static compression, which during the initial ramp phase contains aspects of dynamic compression such as fluid flow and pressure gradients, regulates ERK1/2 and p38. Then we demonstrate that dynamic shear, which induces purely cyclic matrix deformation with little or no fluid flow or pressure gradients, is capable of regulating ERK1/2 and p38 activation in a time-dependent fashion. Finally we show that MAPK activation is necessary for the induction of many genes in response to both compression and shear loading regimens.

EXPERIMENTAL PROCEDURES

Cartilage Preparation and Mechanical Loading—Cartilage explants were harvested from the patello-femoral groove of 1–2-week-old bovine calves as described previously (41). Cylindrical explant disks (3-mm diameter, 1-mm thick) were taken from the middle zone layer, washed with phosphate-buffered saline, and equilibrated for 2–5 days in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 10 mM Hepes buffer, 0.1 mM nonessential amino acids, 20 μg/ml ascorbate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Medium was changed every second day and 16 h before the loading commenced. Anatomically matched explants were placed in 12-well polysulphone loading chambers to allow multiple explants to receive identical loading. One of following loading regimens was then applied (Fig. 1).

Ramp-and-hold compression (6) involved applying a 50% strain (0.5 mm compression) slowly over a period of 5–10 min, and compression was then maintained statically for the indicated duration (Fig. 1, *A* and *F*). Explants, maintained in free swelling conditions, were used as controls for static compression. Dynamic compression (8) involved compressing explants slowly over a period of 5 min to an offset compression of 5%, allowing stress relaxation to occur for an additional 5 min, and then applying a continuous sinusoidal compression (0.1 Hz, 3% strain amplitude in axial displacement control) for the indicated duration (Fig. 1*B*). Explants statically compressed to a 5% offset were used as controls for dynamic compression. Dynamic shear (9) involved maintaining explants at cut thickness and applying a continuous sinusoidal shear strain (0.1 Hz, 3% strain amplitude in angular displacement control) for the indicated duration (Fig. 1, *C* and *E*). Explants maintained at cut thickness were used as controls. For dynamic shear dose-response experiments, anatomically matched cartilage explants were subjected to sinusoidal shear strain amplitudes of 1.5–4.5% at 0.1 Hz for 20 min or to 0% static compression control or free swelling conditions (Fig. 1*D*; 8–10 explants per condition). Upon loading completion, 4–6 explants that received identical loading and inhibitor treatments were pooled and flash-frozen in liquid nitrogen.

Inhibitor Treatment—To investigate the role of MAPK activation, cartilage explants were preincubated with small molecule inhibitors for 1 h before commencement of loading (inhibitors were also present in the medium during loading). U0126 (Sigma) is a highly potent MEK (ERK pathway) inhibitor and was used at 25 μM, a concentration equal to or greater than previously shown to inhibit ERK induced effects in chondrocytes (28, 29, 34, 42). SB203580 (Sigma) is a p38 kinase activity blocker and was used at 20 μM, a concentration equal to or greater than previously shown to inhibit the effects of p38 activation in chondrocytes (31, 43, 44). Four U0126 treated explants, four SB203580 treated explants, and four inhibitor-free explants were placed in a 12-well chamber and subjected to identical loading regimens. For comparison with previous results (6, 8) static compression was applied for 2 or 24 h (Fig. 1*A*), or dynamic compression was applied for 1 or 24 h (Fig. 1*B*), or dynamic shear was applied for 1 or 24 h (Fig. 1*C*). Control explants were subjected to the same inhibitor treatments (see Fig. 1). Three to five experimental replicates were performed for each loading condition.

To assess the impact of protein synthesis during mechanical loading, three separate experiments were performed in which explants were pretreated for 1 h with the protein synthesis blocker cycloheximide (100 μg/ml; Sigma). 50% static compression was then applied for 1, 16, or 24 h to examine the effects of blocking protein synthesis on short and long term mRNA expression (Fig. 1*F*). Explants not treated with cycloheximide were placed in the same loading chambers, and free swelling control explants were subjected to the same inhibitor treatments.

Immunoblotting—After loading, frozen tissue specimens were first pulverized and then homogenized in Nonidet P-40 type homogenization buffer with protease inhibitors (20 mM Tris, pH 7.6, 120 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 40 μg/ml leupeptin) used at 10 μl/mg of tissue. The supernatants were then extracted by rotating at 4 °C for 1 h and centrifuged at 13,000 × *g* for 1 h. Total protein concentration of each supernatant was quantified by the bicinchoninic acid method.

For measurement of ERK1/2 and p38, aliquots containing 20 μg of protein were resolved by 10% SDS-PAGE and were transferred to a nitrocellulose membrane (Schleicher & Schüll). The membranes were blocked with 5% bovine serum albumin in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for

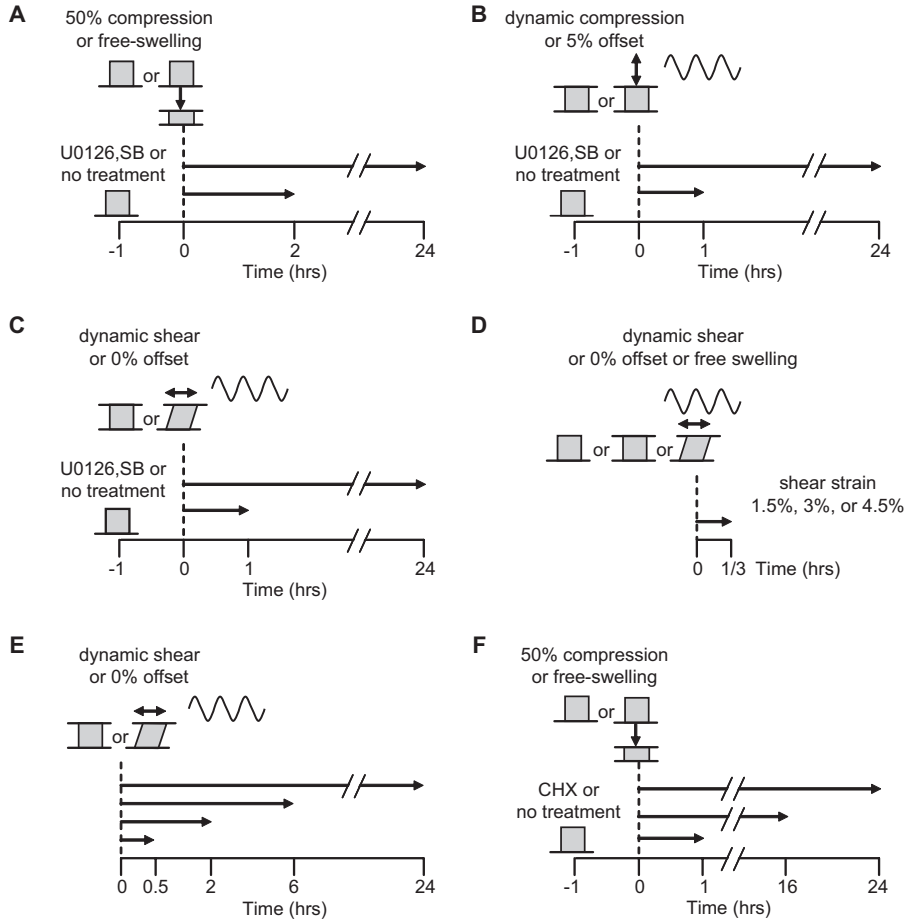


FIGURE 1. **Loading protocols for MAPK inhibitor studies (*A, B, C*), MAPK phosphorylation studies (*D, E*), and cycloheximide studies (*F*).** *A*, a sustained static compression of 50% strain was applied for 2 or 24 h with and without inhibitors to MEK/ERK (25 μM U0126) or p38 (20 μM SB203580) added 1 h prior to loading. Free-swelling explants with identical inhibitor treatment were used as controls. *B*, continuous dynamic compression of 3% strain at 0.1 Hz was applied for 1 or 24 h on top of a 5% static offset, with and without U0126 or SB203580, added 1 h prior to loading. Cartilage explants compressed to 5% strain and with identical inhibitor treatment were used as controls. *C*, continuous dynamic shear of 3% shear strain at 0.1 Hz was applied for 1 or 24 h on top of a 0% offset, with and without U0126 or SB203580, added 1 h prior to loading. Cartilage explants compressed to cut thickness (0% offset) and with identical inhibitor treatment were used as controls. *D*, ERK1/2 phosphorylation shear-dose response. Dynamic shear was applied at 1.5, 3, or 4.5% shear strain for 20 min. Free swelling explants and explants compressed to cut thickness were measured for comparison. *E*, ERK1/2 and p38 phosphorylation time course. Dynamic shear of 3% shear strain at 0.1 Hz was applied for 0.5, 2, 6, or 24 h. In separate experiments static compression of 50% strain was applied for 2 or 24 h. Explants maintained at 0% offset were used as controls. *F*, static compression of 50% strain was applied for 1, 16, or 24 h with cycloheximide added 1 h before loading. Free-swelling cartilage explants with and without cycloheximide treatment were used as controls. *SB*, 203580; *CHX*, cycloheximide.

2 h at 37 °C and incubated with total or phospho-specific anti-ERK1/2 or anti-p38 antibody (1:1000) in TBST (Cell Signaling Technology. Inc.), overnight at 4 °C. Membranes were washed five times in TBST for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature, and then washed in TBST. Phosphorylated ERK1/2 and p38 were detected using enhanced chemiluminescence (Renaissance^{TM} ECL, NEN Life Science Products) and quantified using densitometry.

For measuring MAPKAPK2, aliquots containing 40 μg of protein were resolved by 10% SDS-PAGE and were transferred to an Immun-blot polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature and incubated with total or phospho-specific anti-MAPKAPK2 antibody 1:1000 in blocking solution (Cell Signaling Technology, Inc.) overnight at 4 °C. Membranes were washed three times in TBST for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Inc.) for 1 h at room temperature, and then washed in TBST. Phosphorylated MAPKAPK2 were detected using enhanced chemiluminescence (ECL Plus^{TM}, GE Healthcare).

Real-time PCR—RNA extraction and real-time PCR were performed as described previously (6). Briefly, 4–6 pooled, frozen cartilage explants were pulverized, lysed by blade homogenization in TRIzol reagent (Invitrogen), and separated using phase-gel spin columns (Eppendorf). RNA was purified using the Qiagen RNeasy mini columns with the DNase digest, and 1–2 μg of total RNA was reverse transcribed (Applied Biosystems). Real-time PCR was performed with previously described primers (8) on a MJ Research Opticon 2 using Applied Biosystems SYBR Green reagents. Housekeeping genes (18 S & G3PDH) and control samples were run on every plate for normalization purposes. Cycle threshold levels were converted to relative copy numbers using primer specific standard curves. Expression levels from loaded samples were normalized to the average of the housekeeping genes and also to similarly treated control samples.

Clustering and Statistical Analyses—Our previously described clustering technique was used to place genes with similar expression patterns into a predefined number of groups (6, 8). In brief, the average expression levels of each gene from each loading condition, duration, and MAPK inhibitor treatment were combined into expression vectors forming a data matrix of 17 genes by 18 conditions (3 loading regimens, 2 durations, 3 treatment conditions). Each gene expression vector was normalized by the vector standard deviation to emphasize the pattern of expression rather than overall magnitude. Principal component analysis was then used to reduce dimensionality and visualize the similarity between genes in a three-dimensional space, so that the number of distinct groups could be estimated. The three-dimensional gene coordinates were then iteratively clustered by minimizing the total Euclidean distance

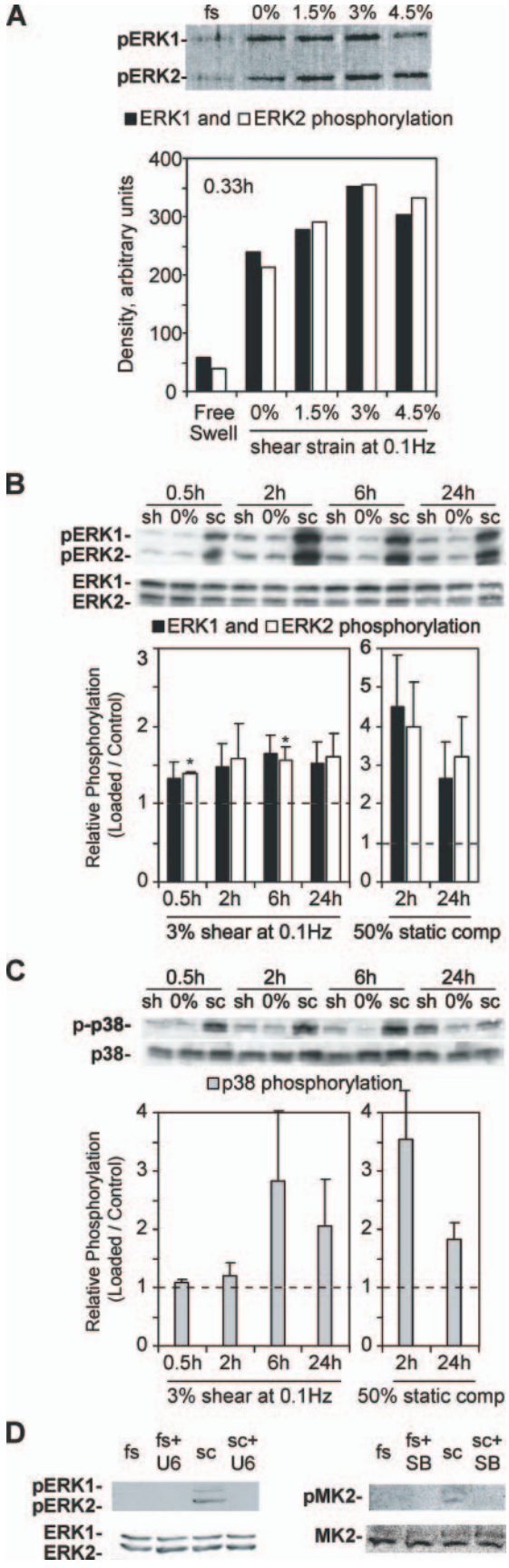


FIGURE 2. **Phosphorylation of ERK1/2 and p38 in response to mechanical loading.** *A*, representative blot and quantified phosphorylated ERK1/2 levels for free swelling (*fs*), 0% shear strain control, and 1.5–4.5% shear strain at 0.1 Hz (20 min of loading). Representative blot and quantified phosphorylated ERK1/2 (*B*) and p38 (*C*) time courses during dynamic shear (*sh*) and static compression (*sc*). Mechanically induced phosphorylation was normalized to 0% control (*dashed line*). Mean + S.E. (ERK, $n = 3$; p38, $n = 2$) is shown. *D*, representative blots of ERK1/2 and MAPKAPK2 (*MK2*) phosphorylation following 1 h of free swelling or 50% static compression in the presence of 25 μM U0126 (*U6*) or 20 μM SB203580 (*SB*). Total ERK1/2, p38, and MAPKAPK2 levels were unchanged in the mechanically loaded or inhibitor-treated samples.

between the genes and corresponding group centroids. A second technique, applying k-means clustering to the entire gene expression vectors, was used to maximize the average gene-to-group centroid correlation. The optimal grouping was chosen based on both Euclidean distance and correlation metrics, and the group centroid was calculated as the average expression profile from the genes within a group. The top five suboptimal groupings were compared with the optimal grouping to ensure that the trends were robust. Distinct separation between group profiles was tested using the three-dimensional group coordinates and Student's *t* test (8).

Comparisons between inhibitor treated and non-treated mechanically induced gene expression levels were performed by two-tailed Student's *t* test. Expression levels from group centroids were similarly compared. Statistical significance was attributed for $p < 0.05$.

RESULTS

MAPK Phosphorylation due to Mechanical Loading—Phosphorylated ERK1/2 levels in free swelling culture, 0% compression static control, and 1.5–4.5% shear strain amplitude at 0.1 Hz for 20 min loading were detected using phospho-specific antibodies. Three experiments for each condition were performed, and a representative blot is shown in Fig. 2*A*. The increase in the phosphorylated ERK1/2 level reached a maximum value at 3% shear strain and slightly decreased at 4.5% shear strain; therefore time course experiments were performed at the 3% shear strain, 0.1 Hz condition. Interestingly, there was a dramatic increase in the phosphorylated ERK1/2 level even at 0% compression over the free swelling condition (Fig. 2*A*). Consistent with our previous study (17), applying 50% static compression for 2 or 24 h resulted in early activation of ERK1/2, decaying to a sustained 2-fold activation by 24 h (Fig. 2*B*). In contrast, 3% dynamic shear induced a consistent 25–50% increase in ERK1/2 activation, compared with activation levels in statically held control explants, over the entire 0.5–24 h period (Fig. 2*B*). 50% static compression increased p38 phosphorylation ~3-fold within 2 h and remained elevated at 24 h (Fig. 2*C*), similar to our previous findings (17). In contrast to the ERK1/2 response to dynamic shear, dynamic shear-induced p38 phosphorylation was delayed, with an observed peak activation of ~2-fold after 6 h and remained elevated at 24 h.

As a control we tested whether MAPK inhibitors could prevent the mechanical loading-induced activation of ERK1/2 and p38 in chondrocytes within cartilage explants. In a previous study it was observed that 1 h of 50% static compression of explants, similar to that used in this study, strongly activated ERK1/2 and p38 (17). We therefore used this loading protocol to test the inhibitory effects of U0126 and SB203580 (Fig. 2*A*).

1 h preincubation with U0126 prior to loading prevented the phosphorylation of ERK1/2 during loading (Fig. 2*D*); preincubation with SB203580 prevented the phosphorylation of MAPKAPK2 (*D*), a major downstream target of p38 (45). Together these results confirm the effectiveness of U0126 and SB203580 in our system.

MAPK Inhibition Alters Mechanically Regulated Gene Expression—Addition of MAPK inhibitors had marginal effects on free swelling gene expression (data not shown). Matrix protein mRNA expression in free swelling explants was up-regulated 10–200% after 2 h of U0126 or SB203580 treatment, but no effect was observed with U0126 treatment after 24 h; 24 h of SB203580 treatment decreased free swelling extracellular matrix (ECM) mRNA expression by 50%. Metalloproteinase mRNA expression in free swelling explants was up-regulated 0–50% after 2 h of U0126 or SB203580 treatment but was generally not affected after 24 h. Basal expression levels of transcription factors c-Fos and c-Jun were not affected by U0126 or SB203580 treatment.

In contrast, mechanically induced matrix protein mRNA levels were markedly affected by MAPK inhibitor pretreatment (Fig. 3*A*). Aggrecan mRNA levels were suppressed to control levels during dynamic loading in the presence of U0126 or SB203580 for both short (1–2 h) and long (24 h) durations (Fig. 3*A*). Mechano-induced up-regulation of type II collagen and link protein was consistently inhibited by U0126; however, SB203580 failed to suppress mechano-induced up-regulation at longer durations, suggesting distinct roles for ERK1/2 and p38 in transducing mechanical stimulation (Fig. 3*A*). Mechano-induced regulation of type I collagen, fibromodulin, and fibronectin mRNA expression was suppressed overall by U0126 and SB203580 during most loading conditions (Fig. 3*A*). The down-regulation of type II collagen, fibromodulin, and fibronectin by 24 h of static compression was partially reversed by the addition of U0126 or SB203580.

Metalloproteinase mRNA was suppressed toward control levels by U0126 and SB203580 under all loading regimens, even during the 1.5- to 10-fold up-regulation induced by dynamic compression and dynamic shear loading (Fig. 3*B*). The only exception was for MMP3 up-regulation induced by 24 h of static compression, which was unaffected by U0216 and enhanced by SB203580. TIMP2 mechano-induced mRNA levels were generally suppressed by the MAPK inhibitors during loading (Fig. 3*B*), whereas TIMP3 mechano-induction was enhanced by U0126 and SB203580 during most time points of static and dynamic compression. However, both MAPK inhibitors clearly suppressed transcription of TIMPs during dynamic shear (Fig. 3*B*).

The mechano-induced up-regulation of transcription factors Sox9, c-Fos, and c-Jun was partially suppressed by U0126 under all loading conditions (Fig. 3*C*). SB203580 also suppressed c-Fos and c-Jun mRNA during dynamic loading, but for static compression only the short-term up-regulation of c-Jun was affected. Sox9 mRNA levels were unaffected by SB203580 except after 1 h of dynamic compression (Fig. 3*C*). Mechano-induced mRNA levels of COX2 and MAPK1 (ERK2) were generally suppressed by MAPK inhibitors (Fig. 3*C*).

Gene Clustering Analysis—Gene clustering was performed to determine the overall effects of inhibiting the MAPK pathway on gene expression levels that were regulated by mechanical loading. Genes were clustered into four statistically distinct groups described in Fig. 4 ($p < 0.02$ for separation between each group). The up-regulation induced by all types and duration of mechanical loading for matrix proteins and proteases in Group 1 were suppressed by treatment with U0126 or SB203580. In addition, Group 1 genes that were down regulated by 24 h of static compression partially returned to control levels by treatment with MAPK inhibitors. Group 2 contained genes that were up regulated to a greater extent after 24 h of dynamic loading, which was suppressed by U0126 and SB203580. During static compression, Group 2 expression levels were suppressed by U0126. Link protein, MMP3, and TIMP3 (Group 3) exhibited distinct transcription profiles in response to different loading conditions and MAPK inhibitor treatment. Although Group 3 was up-regulated 100% in response to dynamic compression, neither U0126 nor SB203580 had an effect on link protein or TIMP3 expression. U0126 suppressed Group 3 after static compression; however, SB203580 enhanced expression of Group 3 genes after 24 h of static compression. Group 4 contained transcription factors c-Fos and c-Jun as well as COX2 and exhibited the largest response to static compression, which was partially suppressed by SB203580 and to a greater extent by U0126. Both MAPK inhibitors suppressed the mechano-regulation of Group 4 in response to dynamic loading with SB203580 being more effective.

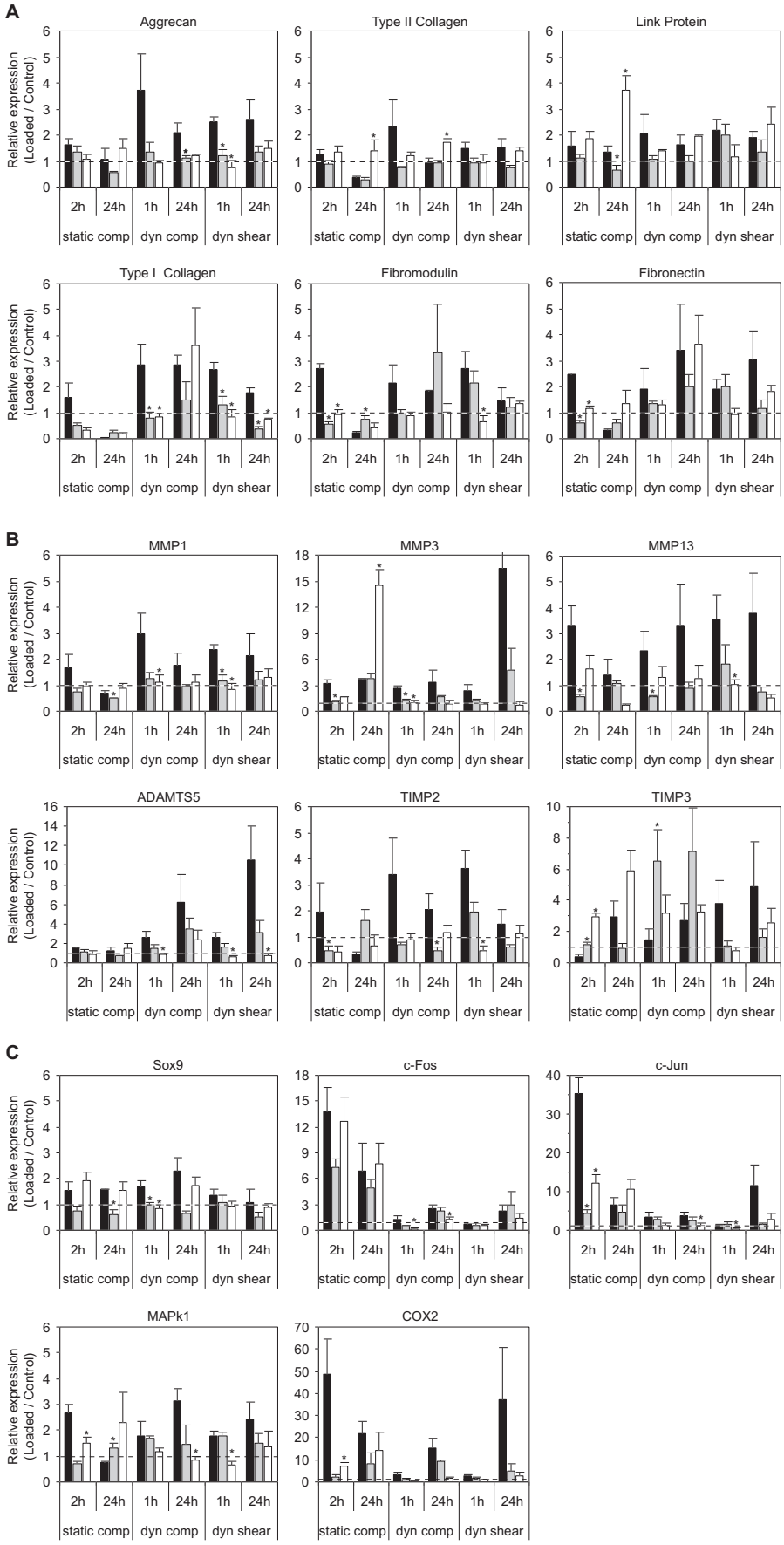
Effect of Cycloheximide Treatment during Static Compression—The inhibition of protein synthesis with cycloheximide greatly affected expression of a number of genes that responded to 50% static compression (Fig. 5). Blocking protein synthesis is known to disturb basal transcription levels (46, 47), and cycloheximide did affect gene expression in free swelling controls: the transcription factors (Sox9, c-Fos, c-Jun) as well as TIMP2, MMP3, and ADAMTS5 increased substantially in cycloheximide-treated free swelling explants compared with untreated controls (data not shown). However, we were particularly interested in how the suppression of protein synthesis would affect static compression-regulated mRNA levels. Static compression transiently induced the up-regulation of aggrecan, type II collagen, and link protein mRNA (consistent with previous studies) (6), which was altered with cycloheximide treatment to become a delayed up-regulation with increasing compression duration. Fibromodulin and fibronectin were unaffected (Fig. 5 and data not shown). The up-regulation of MMP3 and ADAMTS5 by static compression was greatly suppressed by cycloheximide (Fig. 5). Static compression-induced MMP1 and ADAMTS4 levels were partly suppressed, whereas MMP9 and MMP13 were unaffected (data not shown). TIMP3 up-regulation during compression was suppressed by cycloheximide at all time points, and TIMP2 was affected by cycloheximide treatment during longer compression durations (data not shown). Sox9 mRNA levels were suppressed below control levels by cycloheximide treatment during loading (Fig. 5). The large up-regulation of c-Fos and c-Jun by static compression at all time points was completely blocked by cyclo-

heximide treatment (Fig. 5), and the up-regulation of COX2 was blocked at later time points (data not shown).

DISCUSSION

Compression of cartilage *in vivo* induces matrix deformation, hydrostatic pressure gradients and associated fluid flows, and electrical streaming currents. Importantly, compression of cartilage explants *in vitro* has also been shown to cause MAPK activation (17, 18). Here we demonstrate that dynamic shear, which induces cyclic deformation of cells and matrix without associated pressure gradients and flows, is capable of inducing sustained activation of the ERK1/2 and p38 pathways. Furthermore, by applying different mechanical loading regimens known to induce very different effects on chondrocyte biosynthesis and gene expression (*i.e.* static compression *versus* dynamic compression and dynamic shear), we observed that the mechano-regulation of a number of genes involved in cartilage homeostasis was suppressed by MAPK inhibitors, suggesting a prevalent role for MAPK signaling in chondrocyte mechanotransduction pathways.

We observed a dose-dependent increase in ERK1/2 activity in response to dynamic shear (Fig. 2), revealing that isolated cyclic cell and matrix deformation was capable of modestly activating ERK1/2. Much greater ERK1/2 activation was demonstrated in response to continuous 50% strain static compression of cartilage explants (consistent with a previous study) (17). The higher activation of ERK1/2 in response to static compression may therefore be due to the increased strain applied or the transient fluid flows and hydrostatic pressure gradients that were absent during the dynamic shear stimulus (9). Supporting the latter hypothesis, small strain (2.4%) dynamic compression of cartilage explants at lower frequency (0.01 Hz), which would cause intratissue fluid flows and associated pressure gradients, has been reported to



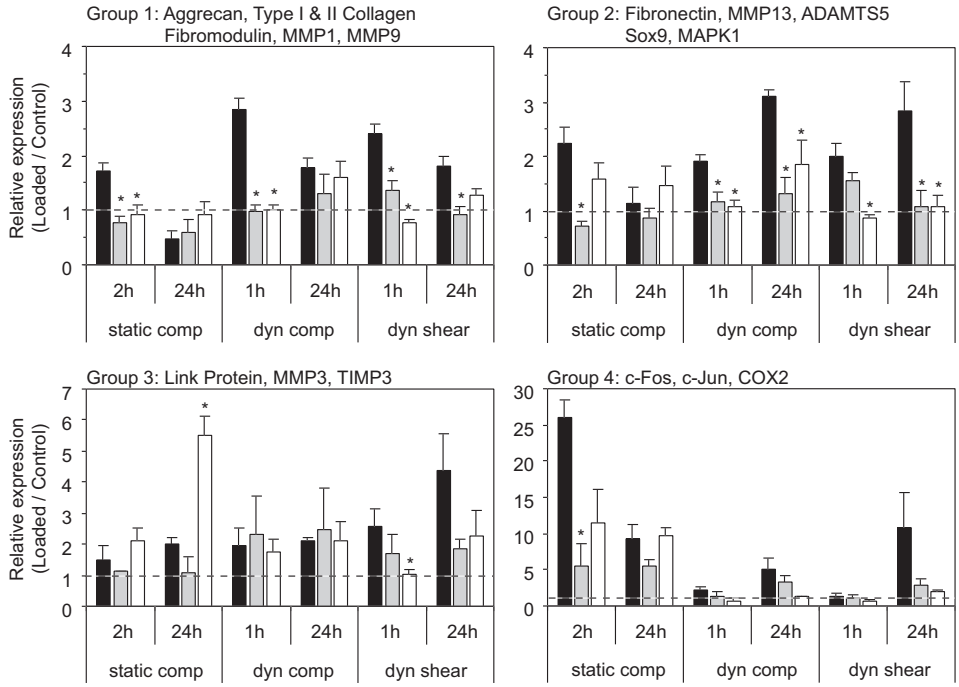


FIGURE 4. **Main expression patterns induced by mechanical loading in the presence of MAPK inhibitors.** *static comp*, 50% static compression (see Fig. 1*A*); *dyn comp*, 3% strain, 0.1 Hz compression (see Fig. 1*B*); *dyn shear*, 3% shear strain, 0.1 Hz shear (see Fig. 1*C*). *Solid column*, no inhibitor; *shaded column*, 25 μM U0126; *open column*, 20 μM SB203580. Group expression levels were calculated from the relative expression levels of the group members (Fig. 3). Mean + S.E. (sample sizes are the number of genes in each group). The corresponding control level is indicated by *dashed line*. *, $p < 0.05$ between no inhibitor and U0126- or SB203580-treated conditions.

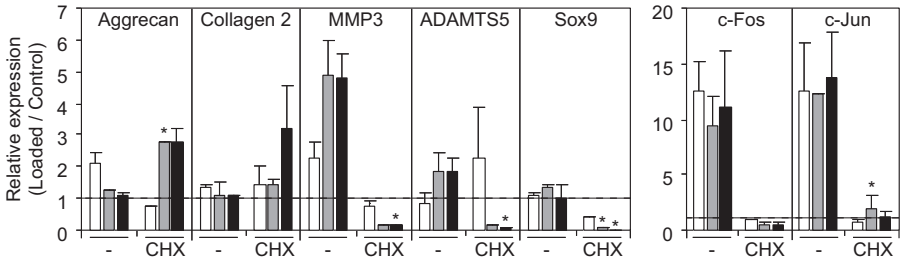


FIGURE 5. **Role of protein synthesis in static compression-induced gene transcription.** Cartilage explants were treated with 100 μg/ml of cycloheximide 1 h prior to 50% static compression. *Open column*, 1 h; *shaded column*, 16 h; *solid column*, 24 h of compression. Expression was normalized to free swelling control levels (*dashed line*). Mean + S.E. ($n = 3$) is shown. *, $p < 0.05$ between cycloheximide (*CHX*) and no inhibitor (−) expression levels.

which mechanical forces could cause MAPK regulation is the cartilage ECM. For example, Li *et al.* (18) showed that phosphorylation of ERK is sustained for up to 16 h in response to IL-1α if chondrocytes are surrounded by an intact ECM; however, in chondrocytes cultured in monolayer, IL-1 transiently activated ERK1/2 within 15 min and returned to basal levels within 1–4 h (28, 30, 32). These findings and recent evidence (50) suggest that the sequestering of signaling factors in the ECM and the presentation of signaling ligands to cell receptors caused by mechanical loading may transduce chondrocyte response to mechanical forces.

Using clustering techniques, we have shown that the activation of ERK1/2 and p38 kinases during mechanical shear and compression has a prevalent role in regulating temporal gene expression patterns (Fig. 4). Inhibition of ERK activation with U0126 or p38 activity with SB203580, during static compression fully or partially, suppressed gene regulation in Groups 1, 2, and 4 (and Group 3 for U0126), suggesting a role for the large, biphasic activation of ERK and p38 seen in our current (Fig. 2*B*) and previous (17) experiments. Inhibition of p38 activity completely suppressed all group expression levels induced during dynamic shear, implicating a function for the 3-fold activation of p38 during long-term dynamic shear. Interestingly, inhibiting ERK also had a general suppressive effect on dynamic shear-induced gene transcription, even though only mild ERK1/2 activation was observed. The widespread suppression of mechanically induced gene transcription by ERK or p38 inhibition has some similarity to our previous observations regarding the effect of chelating intracellular calcium or blocking cAMP activity (6, 8), which suggests these networks may be interconnected in the chondrocyte mechanotransduction pathway.

The common regulatory effects of p38 and ERK to induce downstream signaling may indicate some level of cross-talk in activating downstream transcription factors. One likely candidate is activating protein-1, a heterodimer of c-Fos and c-Jun,

induce a greater than 2-fold sustained increase in ERK1/2 activation (18). In contrast to the ERK1/2 response, static compression caused a transient activation of p38 (Fig. 2, *B* and *C*) (17) and dynamic shear-activated p38 at later time points. Hence, p38 may be differentially sensitive to cyclic matrix deformation compared with ERK1/2.

A numbers of mechanisms have been proposed for how mechanical forces induce proximal biological changes in chondrocytes. For example, stress-activated ion channels (15, 16) and integrin-mediated pathways activated through ECM-cytoskeleton reorganization (48, 49). Another conduit through

FIGURE 3. **mRNA expression levels induced by mechanical loading in the presence of MAPK inhibitors.** *A*, matrix proteins. *B*, matrix proteases and TIMPs. *C*, transcription factors and intracellular signaling molecules. *Solid column*, no inhibitor; *shaded column*, 25 μM U0126; *open column*, 20 μM SB203580. mRNA levels were normalized to control levels (*dashed line*), and in all cases, controls were matched by duration and inhibitor treatment (as described in the legend to Fig. 1). *Static comp*, 50% static compression (see Fig. 1*A*); *dyn comp*, 3% strain; 0.1 Hz compression (see Fig. 1*B*); *dyn shear* = 3% shear strain, 0.1 Hz shear (see Fig. 1*C*). Mean + S.E. ($n = 4$) is shown. *, $p < 0.05$ between no inhibitor and U0126- or SB203580-treated conditions.

known to be downstream of the MAPK pathway (20, 33). It has been shown that gene expression levels of c-Fos and c-Jun dramatically increase within 1 h of a variety of intact cartilage loading regimens (6, 8, 41), and that activating protein-1 binding increases in response to dynamic compression of a tissue engineered cartilage construct (51). Furthermore, we found that c-Fos and c-Jun mechano-regulated mRNA levels were partially suppressed by MAPK inhibition, which also suppressed many matrix proteins and proteases (Fig. 3). Blocking protein synthesis with cycloheximide suppressed the static compression-induced increase in c-Fos and c-Jun mRNA and also altered matrix protein and protease transcription patterns (Fig. 5). Elucidating a causative role for c-Fos and c-Jun will contribute to our understanding of the chondrocyte mechanotransduction pathway.

In chondrocytes, the ERK and p38 pathways have often been associated with catabolic signals (21, 22, 27, 28, 30, 32, 43). Our data, showing that MAPK inhibitors suppressed matrix protein mRNA levels during loading (Fig. 3), support the emerging view that the MAPK pathway may also mediate the responses of anabolic growth factors. For example, transforming growth factor-β-induced aggrecan transcription in ATDC5 chondrogenic cells requires ERK1/2 and p38 activation (42). In addition, p38 signaling contributes toward transforming growth factor-β-stimulated proteoglycan synthesis and chondrocyte proliferation (36, 37), and ERK1/2 is activated by insulin-like growth factor-1, which strongly promotes anabolic chondrocyte behavior (17, 35). It was also shown recently that JNK inhibition during cyclic compression of tissue-engineered constructs prevents up-regulation of aggrecan and type II collagen, as well as MMP3 and MMP13 genes (51). Taken together with our observations of widespread involvement of ERK1/2 and p38 in mechanically induced transcription of matrix proteins, proteases, and TIMPs (Fig. 3), the MAPK pathway may be a central conduit through which mechanical signals regulate anabolic and catabolic activities of chondrocytes.

We have demonstrated that ERK and p38 MAPKs are activated in response to both mechanical shear and compression. In particular, the cyclic matrix and cell deformation produced by dynamic shear regulated ERK1/2 and p38 phosphorylation in a time-dependent fashion. Furthermore, we showed that ERK1/2 and p38 phosphorylation is involved in the time-dependent mechano-regulation of matrix protein, matrix protease, and transcription factor gene transcription. By applying multiple types of mechanical loading, which induce differing biophysical factors and potentially utilize different mechanotransduction pathways, we demonstrated that the MAPK pathway appears to be a critical component of chondrocyte mechanotransduction. However, the differing shear and compression stimuli result in contrasting effects on chondrocyte biosynthesis (3, 4, 9, 10, 12) and gene expression (6, 8). These contrasting effects of mechanical loads may be due in part to different temporal dynamics and magnitudes of MAPK activation, which may lead to load-dependent activation of downstream transcription factors. Alternatively additional signaling pathways may be activated in a load-dependent manner that requires MAPK signaling to induce changes in gene expression. Coupling with additional signaling pathways may also explain how the subtle increase in activation of p38 and ERK1/2 by dynamic shear was capable of dramatically increasing expression of certain genes; however, it should be pointed out that signal transduction pathways are inherently non-linear and thus amplification downstream of p38 and ERK1/2 may also explain the dramatic changes observed. Finally, the downstream processes of transcription, translation, and secretion may also vary specifically with the type of mechanical load applied (52, 53) and warrant further investigation.

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