

Shear and Compression Differentially Regulate Clusters of Functionally Related Temporal Transcription Patterns in Cartilage Tissue^{*§}

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Chondrocytes are subjected to a variety of biophysical forces and flows during physiological joint loading, including mechanical deformation, fluid flow, hydrostatic pressure, and streaming potentials; however, the role of these physical stimuli in regulating chondrocyte behavior is still being elucidated. To isolate the effects of these forces, we subjected intact cartilage explants to 1–24 h of continuous dynamic compression or dynamic shear loading at 0.1 Hz. We then measured the transcription levels of 25 genes known to be involved in cartilage homeostasis using real-time PCR and compared the gene expression profiles obtained from dynamic compression, dynamic shear, and our recent results on static compression amplitude and duration. Using clustering analysis, we determined that transcripts for proteins with similar function had correlated responses to loading. However, the temporal expression patterns were strongly dependent on the type of loading applied. Most matrix proteins were up-regulated by 24 h of dynamic compression or dynamic shear, but down-regulated by 24 h of 50% static compression, suggesting that *cyclic* matrix deformation is a key stimulator of matrix protein expression. Most matrix proteases were up-regulated by 24 h under all loading types. Transcription factors c-Fos and c-Jun maximally responded within 1 h to all loading types. Pre-incubating cartilage explants with either a chelator of intracellular calcium or an inhibitor of the cyclic AMP pathway demonstrated the involvement of both pathways in transcription induced by dynamic loading.

Mechanical forces within synovial joints are known to influence the metabolic behavior of chondrocytes, the sole cell type of cartilage (1–3). Chondrocytes are responsible for the maintenance and turnover of the cartilage extracellular matrix, in

particular the biosynthesis of type II collagen and proteoglycan (aggrecan), which in combination provide the tensile, shear, and compressive stiffness of cartilage. Cartilage thickness and proteoglycan content were found to be enhanced in load-bearing areas *in vivo* (4). Joint inactivity (1, 2) and injury (5) have been found to promote degradation of the extracellular matrix and to reduce cartilage load bearing capacity. The exact mechanisms by which mechanical forces influence the biological activity of chondrocytes are under intense study. In addition, the precise component(s) of the physical stimuli that induce such biological changes following joint movement are not known.

The effects of mechanical loading on cartilage *in vivo* have been studied using intact human and animal cartilage explants *in vitro* to determine the precise biophysical forces and physicochemical changes that result (for reviews, see Refs. 6 and 7). Application of a transient, radially unconfined compressive deformation (using displacement or load control) induces an initial build up of hydrostatic pressure within the tissue and concomitant intratissue fluid flow and flow-induced electrical streaming potentials (Fig. 1A). Mechanical stress relaxation (at constant displacement) or creep relaxation (at constant load) then leads to a new *static equilibrium compressed state* of the tissue at which fluid exudation has ceased (7–9). In contrast, *dynamic compression* leads to cyclic changes in pressure, deformation, and fluid flow within the tissue (Fig. 1B). For the case of unconfined dynamic compression of cylindrical cartilage explant disks using impermeable compression platens, theoretical models have predicted frequency-dependent increases in the dynamic amplitude of the hydrostatic pressure and radial strain at the explant center, with radially directed fluid flow velocities greatest at the explant periphery (10, 11). *Dynamic tissue shear* in the “simple shear” configuration of Fig. 1C induces the cyclic matrix strain in a nearly uniform manner throughout the explant disk with minimal fluid flow or increased hydrostatic pressure (12). Normal joint motion *in vivo* produces a superposition of all these components of cartilage loading.

In vitro loading regimes modulate matrix biosynthesis in a loading-type dependent manner, mimicking *in vivo* responses. Static compression of cartilage explants up to 50% strain inhibited chondrocyte biosynthesis within 1 h, with sustained inhibition for over 24 h (8, 9, 13). In contrast, both types of dynamic loading were found to increase type II collagen and proteoglycan synthesis (12, 13). However, quantitative autoradiography

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§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Appendices A and B, Figs. A1–A7, and Table A1.

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showed that dynamic compression increased proteoglycan synthesis by chondrocytes located mainly near the explant periphery (10) in the region of maximal fluid flow, whereas the stimulatory effects of dynamic shear were observed throughout the explant (12), consistent with the more spatially uniform cyclic shear strain. Studies applying hydrostatic pressure to isolated bovine articular chondrocytes showed that moderate cyclic hydrostatic pressurization (0.5–5 megapascal, 0.1–1 Hz) increased biosynthesis (14, 15), whereas high continuous hydrostatic pressure (>15 megapascal) decreases biosynthesis (14).

Recent studies have focused on intracellular signaling and gene expression pathways that may be linked to the observed changes in biosynthesis caused by mechanical loading of cartilage tissue. Ramp and hold (transient to static) compression of cartilage explants (Fig. 1A) up to 50% strain and for durations of 1–24 h induced time-dependent gene expression patterns that involved intracellular calcium and cyclic AMP (16). Expression of matrix proteins such as aggrecan (17), type II collagen, and link protein (16) were transiently up-regulated but down-regulated by 24 h, and matrix proteases were increasingly up-regulated with the duration of static compression (16). Cyclic hydrostatic pressurization of bovine articular chondrocyte monolayers for 2–24 h, or intermittently for 4 days, increased type II collagen and aggrecan transcription by 3-fold (18, 19). Twenty minutes of cyclic stretching of human chondrocytes cultured on plastic dishes up-regulated aggrecan transcription within 1 h of loading; however, no effect was observed 24 h after loading (20). Cell stretching was also found to induce inositol triphosphate-mediated calcium release in human chondrocytes (21, 22). Constant and cyclic fluid shear flow over bovine articular chondrocyte monolayers cultured on glass slides increased the frequency of intracellular calcium oscillations (23, 24). In cartilage explants, chelating intracellular calcium inhibited the up-regulation of gene transcription induced by static compression in a majority of matrix proteases and proteins (16), notably aggrecan (16, 25).

Although cyclic loading has been shown to affect chondrocyte metabolic activities in cell culture, little is known about the intracellular effects of dynamic loading on intact cartilage explants. Motivated by the widespread induction of gene transcription by static compression (16), we applied loads of varying duration, rate, and magnitude to cartilage explants and measured the response of 25 genes involved in cartilage extracellular matrix homeostasis. To distinguish the effects of matrix deformation from pressure gradients, fluid flow, and streaming potentials, we separately applied dynamic compression and dynamic tissue shear. To identify if certain intracellular signaling pathways were modulated by particular mechanical forces, we used inhibitors of intracellular calcium- and cAMP-mediated signaling during loading. Clustering analysis (16, 26, 27) and principal component analysis (16, 28, 29) were used to group similarly responding genes and to identify the main expression patterns induced by each loading regime. We found that dynamic compression and dynamic tissue shear induced similar expression patterns for anabolic and catabolic genes that were distinct from those induced by static compression. Further-

more, both dynamic loading regimes required intracellular calcium-dependent signaling to induce gene transcription.

EXPERIMENTAL PROCEDURES

Cartilage Specimen Preparation

Cylindrical cartilage explant disks (3 mm diameter, 1 mm thick) were obtained from the middle zone of the patella-femoral groove of 1–2-week-old calves as described previously (8). Explants were washed with phosphate-buffered saline and allowed to equilibrate for 2 to 5 days in low glucose Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 20 $\mu\text{g/ml}$ ascorbate, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ amphotericin, with medium exchanged every second day. To avoid pH or temperature changes at the onset of loading, medium was changed 16 h before loading. Anatomically matched explants were distributed among the loading conditions. In selected experiments, 10 μM BAPTA-AM,⁴ a chelator of intracellular calcium (A.G. Scientific), or 50 μM R_p -cAMP, a competitive inhibitor of cAMP activation of protein kinase A (Sigma), were added to medium 1 h before loading, at concentrations previously shown to suppress compression-induced aggrecan gene transcription (16, 17, 25).

Mechanical Loading and Inhibitor Treatments

Dynamic Compression—For each time point, 12 explants (4 inhibitor-free, 4 BAPTA-AM treated, 4 R_p -cAMP treated) were placed in a polysulphone loading chamber that was mounted into an incubator-housed loading apparatus (30). These explants were dynamically compressed at 0.1 Hz for 1, 4, 8, or 24 h using a 3% strain amplitude superimposed on a 5% static offset strain, loading conditions shown previously to stimulate proteoglycan and protein biosynthesis (8, 13). Minimal liftoff was observed and total harmonic distortion was less than 10% (8). Control explants were statically compressed to 5% strain, based on the initial thickness measurement, for 4 or 24 h.

Dynamic Shear—For each time point, 12 explants (6 inhibitor-free, 6 BAPTA-AM treated) were placed in a polysulphone shear chamber that was mounted into the loading apparatus (30). These explants were maintained at 1-mm cut thickness and dynamically sheared at 0.1 Hz for 1, 4, or 24 h using a 3% engineering shear strain amplitude. This resulted in an application of cyclic shear deformation with less than 10% total harmonic distortion (12). These tissue shear loading conditions were shown previously to produce minimal fluid flow and to stimulate proteoglycan and protein biosynthesis (12). Over the course of 5 days, explants swell up to 20%. Therefore, to minimize unwanted compressive loading, all dynamic shear experiments were performed on day 2, where swelling of only 0–10% had occurred. Control

⁴ The abbreviations used are: BAPTA-AM, bis-(aminophenoxy)ethanetetraacetic acid acetoxymethyl; PCA, principal component analysis; TIMP, tissue inhibitor of matrix protease; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; COX-2, cyclooxygenase-2; TGF, transforming growth factor; TNF α , tumor necrosis factor α ; NF κ B, nuclear factor κ B.

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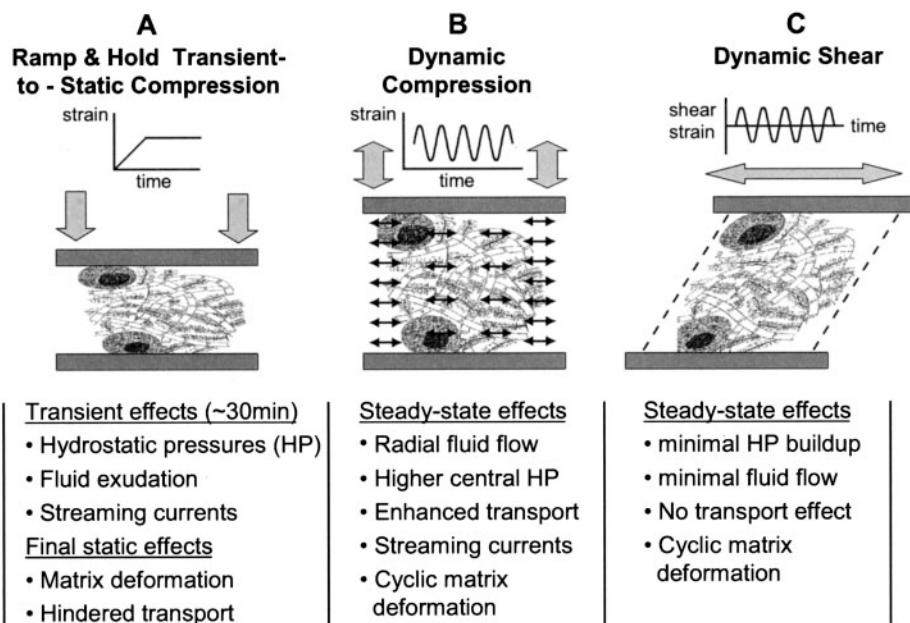


FIGURE 1. Fields, forces, and flows, induced by static and time varying mechanical stimuli. Adapted from Ref. 7.

explants were maintained at cut thickness for 4 or 24 h within similar chambers but not dynamically loaded. Immediately upon loading completion, cartilage explants subjected to identical mechanical loading and inhibitor treatment were pooled, flash frozen in liquid nitrogen, and stored at -80°C .

RNA Extraction and Real-Time PCR

Pooled cartilage explants were disrupted with a liquid nitrogen-cooled tissue pulverizer, placed in a TRIzol lysis buffer (Invitrogen) and lysed using a tissue homogenizer (BioSpec Products Inc). The lysate was separated using phase-gel spin columns (Eppendorf) spun at $10,000 \times g$ for 10 min, and the supernatant was transferred to Qiagen RNeasy Mini columns. Total RNA was purified according to the manufacturer's protocol with the on-column DNA digest. Approximately $1-2 \mu\text{g}$ of total RNA was reverse transcribed (Applied Biosystems) on an Eppendorf Mastercycler. Real-time PCR was performed on an MJ Research Opticon 2 machine using Applied Biosystems SYBR Green master mixture. Primers were designed for the genes listed in supplemental Table A1, tested, and calibrated as previously described (16). Mechanically loaded samples were always run on the same PCR plate as unloaded controls and housekeeping genes 18S and glyceraldehyde-3-phosphate dehydrogenase were run on every plate. Cycle threshold values were converted to relative copy numbers using primer-specific standard curves. To normalize for starting cDNA content, copy numbers were divided by the average of the mean-centered housekeeping genes for each sample on a plate. Relative gene expression levels were calculated by dividing dynamically loaded sample expression levels by control expression levels.

Clustering and Statistical Analyses

To elucidate the main expression patterns induced by dynamic loading, and to determine subsets of genes responding to the same signaling events during loading, the following gene clustering procedure was performed. For each gene the expression levels from the inhibitor free, BAPTA-AM, and R_p -cAMP treatments were combined into a single gene expression vector time course (R_p -cAMP for dynamic compression only). To emphasize expression vector shape rather than amplitude, each vector was standardized by the vector standard deviation as described previously (16), and clustered using two different clustering techniques. First, principal component analysis (PCA) was applied to the data matrix that contained all standardized gene vectors from either dynamic com-

pression or shear to determine the main principal components (similar to eigenvectors). The projection of the standardized vectors along the three main principal components was used to create a three-dimensional representation of the data that enabled the number of groups to be determined graphically (see Ref. 16 for more detail). Euclidean distance k means clustering of the three-dimensional gene projection coordinates was then performed to group the genes. A second technique applying correlation-based k means clustering to the full-length standardized expression vectors was also used to ensure reliable clustering. Centroids were calculated as the average of the standardized gene expression vectors within a group. To determine whether distinct expression patterns had been found, Student's t test between group centroid vectors was performed using the Euclidean distance between the centroid vectors and the variance of each centroid (calculated as the average gene-to-centroid norm for all genes within a group). To confirm the gene groups were robust, the top five ranked groupings were examined, as well as varying the number of total groups. A complete mathematical description of the clustering and statistical approaches is in supplemental Appendix A.

In an effort to further elucidate the effects of mechanical forces on gene transcription, expression vectors containing the inhibitor-free 1-, 4-, and 24-h time points of previously published 50% static compression expression levels (16) were combined with dynamic compression and dynamic shear inhibitor-free time courses. Clustering analysis was performed as described above. Group and gene expression levels were compared with the normalized control level of 1 using Student's t tests; a p value <0.05 was considered significant.

RESULTS

Effects of Dynamic Compression—Dynamic compression of cartilage explants generally up-regulated many genes in a time-

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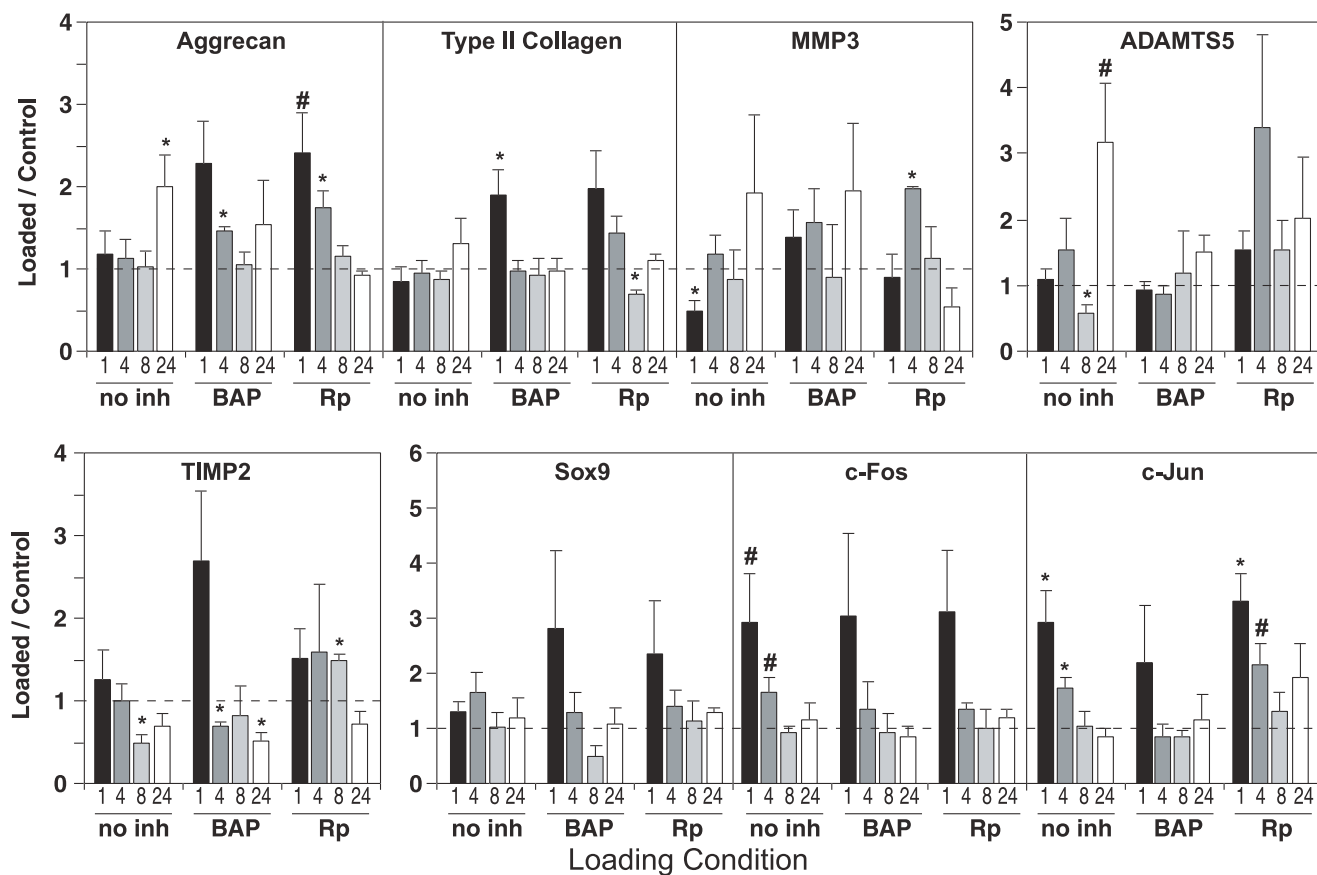


FIGURE 2. Selected gene expression profiles induced by dynamic compression (3% strain, 0.1 Hz). Conditions include dynamic compression time courses from inhibitor-free (*no inh*, $n = 7$ experiments), BAPTA-AM pretreatment (*BAP*, $n = 5$ experiments), or R_p -cAMP pretreatment (R_p , $n = 4$ experiments). Each experiment was from a separate animal, and for each experiment four similarly treated explants were pooled for RNA extraction for each time point and treatment combination. Expression levels are normalized to those of control explants that were compressed to 5% strain (*dashed line*). *Solid column*, 1 h; *dark shaded column*, 4 h; *light shaded column*, 8 h; *open column*, 24 h. Mean \pm S.E. *, $p < 0.05$; #, $p < 0.07$ only compared with the equivalently treated control level of 1.

dependent manner (expression levels for all genes are shown in supplemental Appendix A and Figs. A1 and A2). Transcription of matrix proteins aggrecan and type II collagen (Fig. 2) was affected by long term dynamic compression with increases of 30–100% after 24 h. Link protein, type I collagen, fibromodulin, and fibronectin mRNA levels responded in a similar way (supplemental Fig. A1). The expression of COX-2 and most matrix proteases increased by 100–200% by 24 h, although MMP3, MMP9, MMP13, and COX-2 were mainly suppressed during earlier time points (Figs. 2 and supplemental A1 and A2). Of the TIMPs, only TIMP1 was up-regulated by dynamic compression (Fig. A1). Immediate early genes *c-Fos* and *c-Jun* and signaling genes mitogen-activated protein kinase-1 (MAPK1), and TNF α were transiently up-regulated by 100–200% after 1 h, returning to control levels after 8 h of loading (Figs. 2 and supplemental A2). TGF β and ribosomal 6-phosphate were unaffected by dynamic compression (supplemental Fig. A2). Sox9, interleukin 1 β , and HSP70 were mildly up-regulated only at the 4-h time point (Figs. 2 and supplemental A2), although HSP70 was scarcely detected even during loading and, hence, was not included in the clustering analysis.

When the inhibitor-free, BAPTA-AM treated, and R_p -cAMP-treated gene expression levels were combined, PCA identified

three major principal components that account for over 75% of the variance in the data set. Identical gene groupings were found using either PCA or correlation based clustering techniques producing three statistically distinct (p values < 0.02) and robustly conserved groups (Fig. 3 and Table 1). (Prior to clustering HSP70 and MMP9 were removed as PCA analysis identified their gene expression profiles as very different from all other genes.) Group C1 contained a number of matrix proteins and was characterized by up-regulation after 24 h of dynamic compression. The presence of BAPTA-AM suppressed the long term up-regulation of Group C1 genes, but enhanced expression only at earlier time points (1 and 4 h). Similarly R_p -cAMP treatment suppressed long term up-regulation of Group C1 but enhanced short-term expression. Group C2 was mainly composed of proteases that were suppressed during early time points, up-regulated by 24 h of loading, and generally restrained to controls levels when BAPTA-AM was present (Fig. 3). R_p -cAMP treatment generally up-regulated Group C2 expression, especially COX-2. Group C3 contained transcription factors *c-Fos* and *c-Jun* and other signaling molecules that exhibited transient up-regulation by dynamic compression, which was partially suppressed by BAPTA-AM and unaffected by R_p -cAMP treatment during loading (Fig. 3).

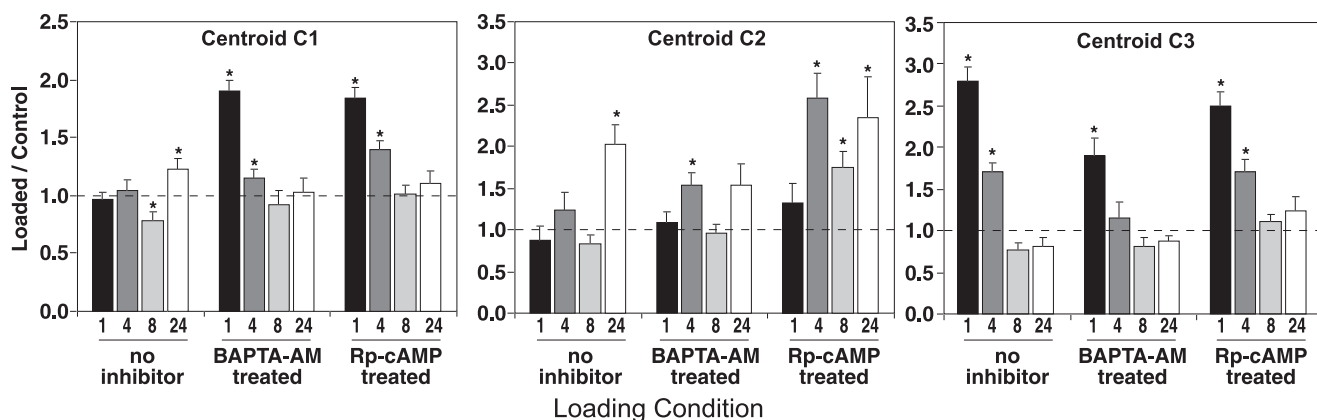


FIGURE 3. Main expression trends induced by dynamic compression (3% strain, 0.1 Hz) with and without pretreatment with inhibitors of the intracellular calcium or cAMP signaling pathways. Centroid expression vectors were calculated by averaging the standardized gene expression vectors in each group. Group gene members are listed in Table 1. Expression levels are normalized to those of control explants that were maintained at the static offset strain of 5% strain (dashed line). Solid column, 1 h; dark shaded column, 4 h; light shaded column, 8 h; open column, 24 h. Mean \pm S.E. (sample sizes are the number of genes in each group listed in Table 1). *, $p < 0.05$ compared with the equivalently treated control level of 1.

TABLE 1
Main groups found by clustering gene expression profiles

Loading conditions	Groups ^a	Genes clustered
Dynamic compression \pm BAPTA-AM or R_p -cAMP	C1	Aggrecan, Type I collagen, Type II collagen, link protein, fibromodulin MMP1, TIMP2, TIMP3, Sox9, IL-1 β , TGF β , ribosomal 6-P
	C2	Fibronectin, MMP3, MMP13, ADAMTS4, ADAMTS5, TIMP1, COX-2
	C3	c-Fos, c-Jun, MAPK1, TNF α
Dynamic shear \pm BAPTA-AM	S1	Aggrecan, Type II collagen, link protein, fibromodulin, fibronectin MMP1, MMP3, MMP13, TIMP3, MAPK1, COX-2, ribosomal 6P
	S2	Type I collagen, MMP9, ADAMTS4, Sox9, c-Fos, c-Jun, TNF α , TGF β
	S3	ADAMTS5, TIMP1, TIMP2, interleukin 1 β
Static compression \pm BAPTA-AM or R_p -cAMP (data from Ref. 16)	ST1	Aggrecan, Type II Collagen, c-Fos, c-Jun
	ST2	Link protein, fibromodulin, MMP1, TIMP2, Sox9, MAPK1
	ST3	Type I collagen, MMP3, MMP9, MMP13, TIMP1, ribosomal 6-P
	ST4	ADAMTS4, ADAMTS5, fibronectin, TIMP3, COX-2, HSP70, TGF β
Static compression, dynamic compression, or dynamic shear (1, 4 and 24 h only)	M1	Aggrecan, Type II collagen, link protein, fibromodulin, MMP1, TIMP3
	M2	Type I collagen, MMP3, MMP9, MMP13, ADAMTS4, TNF α , COX-2, ribosomal 6-P
	M3	c-Fos, c-Jun
	M4	ADAMTS5, TIMP1, TIMP2, Sox9, MAPK1, TGF β

^aGroupings for each set of experiments were found by iteratively clustering gene expression profiles based on correlation and Euclidean distance metrics. Final groups were compared with the top five suboptimal groupings to ensure trends were robust. All groups within each set of experiments were found to be statistically significantly separated according to Student's *t* tests performed on group centroid-to-centroid distances. Full mathematical details are in supplemental materials Appendix A.

Effects of Dynamic Tissue Shear—Dynamic shear had a markedly pro-anabolic effect on gene transcription (all gene expression profiles are shown in supplemental Appendix A and Figs. A3 and A4). In particular, aggrecan, type II collagen, link protein, fibromodulin, and fibronectin were all up-regulated 1.5–5.4-fold by 24 h; aggrecan was up-regulated almost 100% after 1 h (Fig. 4). Interestingly, Sox9 was transiently up-regulated by almost 100% after 1 h (Fig. 4). Matrix proteases and COX-2 were up-regulated after 24 h with MMP1 up-regulated 4-fold and MMP3 up 1.5-fold (Figs. 4 and supplemental A3). The TIMPs were up-regulated by 50–200% at varying time points (Figs. 4 and supplemental A3). Similar to dynamic compression, c-Fos and c-Jun were transiently up-regulated by 2–3-fold within 1 h in response to dynamic shear, and c-Jun was also up-regulated by 2-fold after 24 h (Fig. 4). TGF β and interleukin 1 β were mildly transiently up-regulated at early time points, and ribosomal 6-phosphate was unaffected (supplemental Fig. A4). MAPK1 was up-regulated \sim 150% after 1 and 24 h (supplemental Fig. A4).

The main three principal components found by PCA of the dynamic shear-stimulated inhibitor-free and BAPTA-AM-treated expression profiles accounted for 89% of the data variance, and clustering analysis highlighted three statistically distinct (p values < 0.05) and robustly conserved expression patterns (Fig. 5, Table 1). Group S1 contained a majority of the matrix proteins and proteases (Fig. 5, Table 1) that were up-regulated by long term (24 h) dynamic shear; most of these genes were also up-regulated by 24 h of dynamic compression (Fig. 3), although shear led to an even greater effect. In contrast to dynamic compression, the presence of BAPTA-AM suppressed expression of matrix proteins and proteases during dynamic shear to control levels at all time points (no enhancement was observed after 1 h). Hence, matrix proteins and proteases grouped together in response to dynamic shear, whereas matrix proteins and proteases grouped separately in analysis of dynamic compression. Group S2 contained genes that transiently responded to dynamic shear and were partially suppressed when BAPTA-AM was present, similar to Group C3.

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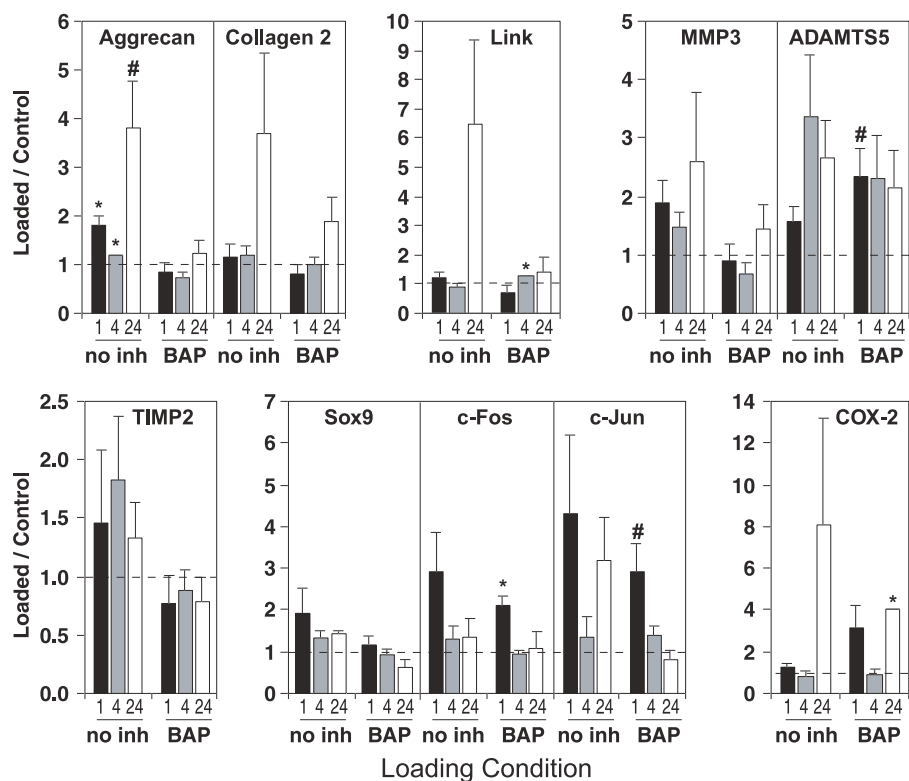


FIGURE 4. Selected gene expression profiles induced by dynamic shear (3% shear strain, 0.1 Hz) without (*no inh*, $n = 4$ experiments) and with BAPTA-AM treatment (*BAP*, $n = 4$ experiments). Each experiment was from a separate animal, and for each experiment six similarly treated explants were pooled for RNA extraction for each time point and treatment combination. Expression levels are normalized to those of control explants that were maintained at cut thickness (*dashed line*). *Solid column*, 1 h; *shaded column*, 4 h; *open column*, 24 h. Mean \pm S.E. *, $p < 0.05$; #, $p < 0.07$ only compared with the equivalently treated control level of 1.

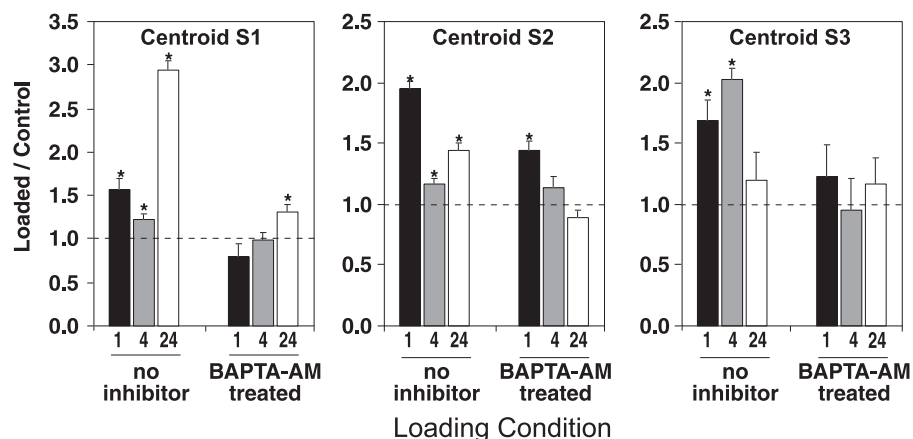


FIGURE 5. Main expression trends induced by dynamic shear (3% shear strain, 0.1 Hz) with and without BAPTA-AM pretreatment. Centroid expression vectors were calculated by averaging the standardized gene expression vectors in each group. Group gene members are listed in Table 1. Expression levels are normalized to those of control explants that were maintained at cut thickness (*dashed line*). *Solid column*, 1 h; *shaded column*, 4 h; *open column*, 24 h. Mean \pm S.E. (sample sizes are the number of genes in each group listed in Table 1). *, $p < 0.05$ compared with the equivalently treated control level of 1.

Genes in Group S3 had a slower transient response to dynamic shear that was suppressed by the presence of BAPTA-AM. Group S3 had the lowest individual gene to centroid correlations indicating that the genes were only loosely grouped, probably based on their poor correlation to the genes in other groups.

Effects of Mechanical Loading Type on Gene Expression—To compare the effects of different mechanical loading regimes on

chondrocyte gene expression, the 1-, 4-, and 24-h inhibitor-free time points from the dynamic compression and dynamic shear experiments were combined with previous data from cartilage explants that were compressed over a 3-min interval to a final strain of 50% and held for periods of 1, 4, and 24 h (16). The expression levels for all genes are included in the supplemental Appendix A (supplemental Figs. A5 and A6). Clustering analysis revealed that four principal components were necessary to represent the data. The added complexity of the dataset was evidenced by a minimum of four groups being required for the two clustering techniques to produce similar findings, which resulted in identical groupings. All four centroids were found to be significantly separated from each other (p values < 0.03) and statistically more correlated than randomly grouped genes (Table 1, Figs. 6 and supplemental A7). Group M1 contained aggrecan, type II collagen, link protein, fibromodulin, MMP1, and TIMP3, which were transiently up-regulated 50% by static compression but inhibited by 24 h (Fig. 6). Group M1 genes were also up-regulated $\sim 50\%$ by 24 h of dynamic compression and 3.5-fold by 24 h of dynamic shear. Most Group M1 genes were in Groups C1 and S1. Group M2 contained type I collagen, matrix proteases MMP-3, -9, -13, and ADAMTS4, as well as TNF α , COX-2, and ribosomal 6-phosphate, which were up-regulated by long term loading by all three protocols. Static compression induced the greatest up-regulation of Group M2, 3-fold after 24 h, and both dynamic compression and dynamic shear induced milder up-regulation of $\sim 50\%$ (Fig. 6). Dynamic shear for 1 h also up-regulated Group M2 genes. c-Fos and c-Jun formed Group M3 and were

characterized by transient up-regulation in response to all loading protocols (Fig. 6). Static compression induced the greatest up-regulation in Group M3 (>6 -fold), which remained partially up-regulated after 24 h; dynamic compression and dynamic shear produced smaller increases (2-fold) and mainly at the 1-h time point. Group M4 contained genes from Group S3 and were only responsive to dynamic shear (Fig. 6).

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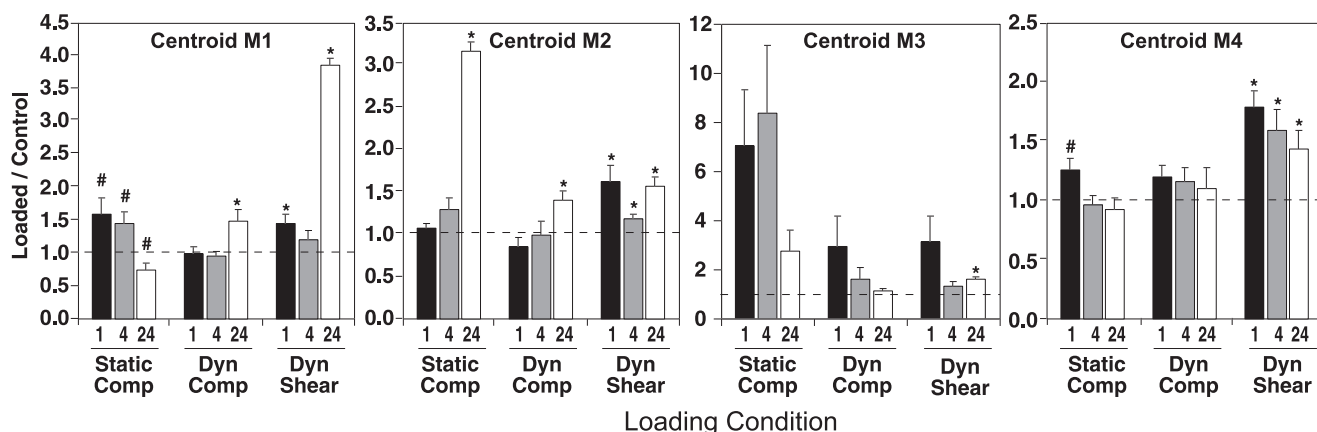


FIGURE 6. Main expression trends induced by static compression, dynamic compression, and dynamic shear. Centroid expression vectors found by averaging the standardized gene expression vectors in each group. Group gene members are listed in Table 1. Expression levels are normalized to corresponding control explants (dashed line). Solid column, 1 h; shaded column, 4 h; open column, 24 h. Mean \pm S.E. (sample sizes are the number of genes in each group listed in Table 1). *, $p < 0.05$. #, $p < 0.07$ only compared with the equivalently treated control level of 1.

DISCUSSION

During joint loading *in vivo*, cartilage experiences a combination of shear and compressive deformation having both sustained (static) and cyclic components (*i.e.* walking). These tissue motions result in a complex combination of intratissue fluid flows, pressure gradients, and extracellular matrix deformations (7, 10). We have used an *in vitro* loading model incorporating dynamic shear and dynamic unconfined compression of tissue explant disks; whereas such *in vitro* models cannot exactly duplicate the complexities of *in vivo* loading, they can help to simulate and isolate the relative effects of these intratissue physical forces on cellular behavior. Our study aimed at examining the individual components of *in vivo* mechanical forces by investigating three loading regimes: dynamic compression, dynamic shear, and static compression. We found that 24 h of dynamic compression, associated with poroelastic matrix deformation and fluid flow, up-regulated matrix protein and protease transcription (Fig. 6). Dynamic shear, in the absence of fluid flow or hydrostatic pressure gradients, also induced matrix protein transcription highlighting the importance of cyclic matrix deformation in chondrocyte mechanotransduction. In contrast, after 24 h of higher magnitude static compression, matrix proteins were down-regulated, whereas matrix proteinases were highly up-regulated (16). By employing clustering algorithms and dimensional reduction techniques we were able to examine the time course expression profiles induced by dynamic compression, dynamic shear, and static compression and show that chondrocytes differentially regulate functional subsets of genes in response to mechanical forces. In addition, clustering analysis revealed that most genes examined required intracellular calcium dependent signaling to respond to mechanical stimuli.

Dynamic loading has been shown previously to be an anabolic stimulus for major cartilage matrix proteins aggrecan and type II collagen. In the present study, clustering analysis showed that dynamic compression and dynamic shear up-regulated transcription of matrix proteins particularly after 24 h (Group C1, Fig. 3 and Group S1, Fig. 5). The similarity in matrix expression profiles suggests that cyclic matrix deformation is the essential extracellular component of mechanical loading

that up-regulates matrix protein gene transcription. Our observations parallel discoveries at the protein level in explant, tissue engineering, and cell-based studies. Cyclic matrix deformation that led to an increase in matrix gene transcription was previously found to increase proteoglycan and type II collagen synthesis in comparable cartilage explants subjected to similar loading protocols (10, 13, 31) and to enhance mechanical properties of tissue engineered cartilage implants (32–34). Dynamic shear, which increased transcription of matrix proteins to a greater extent than dynamic compression, also increased matrix biosynthesis, preferentially collagen synthesis (12). Cyclic stretching of isolated chondrocytes increased aggrecan gene expression (20), perhaps through stretch deformation of the cell membrane.

Interestingly we have found that dynamic loading also stimulates matrix protease transcription. This is consistent with recent studies showing that dynamic compression of chondrocyte-seeded soft fibrin gels (35) and peptide scaffolds (34) caused an increased in glycosaminoglycan loss along with an increase in glycosaminoglycan synthesis during long term culture. The increase in glycosaminoglycan loss in peptide gel culture was associated with an increase in aggrecanase cleavage of aggrecan core protein (36), consistent with our observations of dynamic compression-induced transcription of ADAMTS4 and ADAMTS5 (Figs. 2 and supplemental Fig. A1). We also observed increased transcription of MMP3 during dynamic compression and mild increases in MMP9 and MMP13 transcription (Figs. 2 and supplemental Fig. A1). Similarly, dynamic compression of bone cartilage explants was found to up-regulate MMP2 and MMP9 protein expression and protease activity for 24 h (37). Interestingly COX-2, a known stimulator of PG degradation via prostaglandin E2 (38) and activator of matrix proteases (38), always clustered with a majority of the proteases (Figs. 3, 5, and 6) and thus may be part of the mechanotransduction signaling pathway. In our experiments long term dynamic shear also increased matrix proteases and COX-2 transcription (Fig. 4 and supplemental Figs. A3 and A4). Therefore, a study of the long term effects of cyclic loading on matrix protease activity is necessary to confirm the anabolic benefits.

To further interpret the influence of mechanical loading

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type, we clustered the inhibitor-free time courses of static compression, dynamic compression, and dynamic shear into four statistically distinct group expression patterns (Fig. 6). Interestingly the groups contained functionally related genes, suggesting that mechanical forces differentially affect subsets of genes in a functionally coordinated manner. For example, Group M1 contained 5 of the 6 matrix proteins and was up-regulated after long term loading of both dynamic protocols but was only transiently increased in response to static compression. Group M1 may be considered an anabolic or high turnover group, with MMP1 and TIMP3 (which always clustered with these matrix proteins, Figs. 3, 5, and 6) perhaps acting as the counterbalancing components of this group. Group M1 highlights the responsiveness of matrix protein transcription (and synthesis (12, 13)) to the time varying nature of mechanical stimuli, and the strong similarity between dynamic compression and dynamic shear responses further supports the hypothesis that matrix deformation is the key biophysical regulator of matrix proteins during loading. In contrast, Group M2 was up-regulated in response to all mechanical loading regimes by 24 h (particularly static compression) and contained mainly matrix proteases, COX-2, and type I collagen, and can be considered a catabolic group. c-Fos and c-Jun (Group M3) transiently responded to all types of mechanical loading tested (Figs. 3, 5, and 6) returning to control levels by 24 h. Certain genes responded greatest to the dynamic shear stimulus (Group M4).

The discovery of functional related, co-expressed genes suggests that distinct transcription pathways are activated by mechanical forces. For example, the milder stimulation of c-Fos and c-Jun (Group M3) in response to the dynamic loading compared with static loading may be related to the lower amplitude of the initial deformation applied to the cartilage in dynamic loading. c-Fos and c-Jun are downstream of the mitogen-activated protein kinases (39), which have been identified as mechanically sensitive to static compression (40, 41). Extracellular signal-regulated kinases 1 and 2 become maximally phosphorylated within 10 min of application of a 50% static compression, indicating an immediate response to the changing environmental conditions (40). The mRNA levels of c-Fos and c-Jun (also known as immediate early genes) have been observed to transiently increase >40-fold after a mechanical injury stimulus of 50% compression at a strain rate of 1 mm/s (42). Therefore the onset of mechanical loading, which induces matrix deformation, fluid exudation, and increased hydrostatic pressure may be the general stimulatory signals for c-Fos and c-Jun. Interestingly many catabolism-related molecules (Group M2) also maximally responded to static compression at later time points. We examined the promoter regions of Group M2 genes and found over-representation of the binding sites for transcription factor activating protein-1 (see supplemental Appendix B). Activating protein-1 is a heterodimer of c-Fos and c-Jun and along with NF κ B (which was also over represented) is involved in cytokine induction of matrix proteases in chondrocytes (43, 44), supporting a connection between Groups M2 and M3. A binding site for the glucocorticoid receptor, which suppresses activating protein-1 activity (45), was also identified in the promoter region of genes in Group M2 possibly serving to counterbalance the regulation of this group. Therefore, one

could speculate that the transient up-regulation of transcription factors c-Fos and c-Jun in response to the onset of loading regulates the matrix proteases at later time points.

To understand the signaling pathways involved in cartilage mechanotransduction we examined transcriptional responses to mechanical loading in the presence of intracellular calcium and cAMP pathway inhibitors. Chelating intracellular calcium while applying dynamic compression suppressed the transcription of matrix proteins (Group C1, Fig. 3), and partially inhibited matrix proteases (Group C2), c-Fos, and c-Jun (Group C3), consistent with our previous observations for static compression (16). Physical stimuli occurring during dynamic compression of cartilage explants, such as fluid shear, cell stretching, and mechanical compression have all been found to increase the intracellular calcium concentration in chondrocytes (46–48). Furthermore, increased intracellular calcium concentrations via release from intracellular stores is required for fluid shear-induced glycosaminoglycan synthesis (49) and compression-induced aggrecan gene transcription (25). The main expression trends induced by dynamic shear were also suppressed by intracellular calcium chelation (Fig. 5), suggesting that the effects of pure cyclic matrix deformation are also mediated by intracellular calcium-dependent signaling. Therefore, intracellular calcium-dependent signaling is a key event through which a variety of mechanical forces influence chondrocyte behavior. In recent experiments (50), it was also found that fluid flow-induced increase in intracellular calcium concentration was not responsible for aggrecan gene up-regulation; however, increases in cytosolic calcium by other methods did induce aggrecan gene up-regulation. In contrast to the essential role of intracellular calcium-dependent signaling pathways in chondrocyte mechanotransduction, we found that blocking cAMP-dependent signaling by R_p -cAMP during dynamic compression did not entirely abolish mechano-induced transcription.

We have shown that time varying mechanical loads, comparable with those experienced *in vivo*, regulate the transcriptional behavior of chondrocytes. Genes encoding proteins with similar functions responded to the mechanical loading in a coordinated manner, suggesting that mechanical forces differentially regulate functional subsets of genes. These discoveries suggest that a proteomics study of multiple mechanical loading regimes would be valuable, especially to determine whether a similar clustering pattern will exist between translational and transcriptional expression profiles. It will also be important to confirm the role of species and age on these responses (51, 52), and to determine whether this behavior is present in osteoarthritic cartilage where the chondrocyte mechanotransduction pathway is altered. Degradation of cartilage after injury or during osteoarthritis could modify the process through which forces are transduced into biological responses, altering which sets of genes become up-regulated, and thus changing the balance between anabolic and catabolic processes. There is now substantial evidence of a key role for intracellular calcium-dependent signaling in mechanically induced chondrocyte gene expression; however, further work is required to determine how calcium is mobilized to initiate calcium-dependent signaling. The consistent, transient response of transcription factors

c-Fos and c-Jun to mechanical loading motivates ongoing studies to examine the extent to which the mitogen-activated protein kinase pathway is involved in mechano-induced gene expression.

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