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The Proteoglycan Metabolism of Mature Bovine Articular Cartilage Explants Superimposed to Continuously Applied Cyclic Mechanical Loading

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This study describes the effect of load magnitude, frequency and duration on proteoglycan (PG) biosynthesis and loss in mature bovine articular cartilage explants. Cultured full thickness cartilage discs were subjected to a continuously applied, uniaxial compressive cyclic load. The loads were applied using a sinusoidal waveform of 0.001, 0.01, 0.1 or 0.5 Hz-frequency and a peak stress of 0.1, 1.0, 2.5, or 5.0 MPa for a period of 1, 3 or 6 days. Increasing the load magnitude, as well as the duration of loading, reduced the PG biosynthesis. Reducing the load frequency abolished the inhibitory effect of a given load magnitude on PG biosynthesis, even though explants were more compressed. Increasing the load magnitude stimulated the release of newly synthesized PGs from explants, whereas an elevated duration of loading significantly decreased the release of endogenous PGs. Explants loaded for 1 or 3 days were viable as determined biochemically, whereas 6 days of loading resulted in a slightly diminished viability of explants. This study demonstrates that the duration and intensity of loading influences the inhibition of PG biosynthesis, while PG loss is only modulated by the magnitude and duration of loading.

Articular chondrocytes are capable of successfully maintaining articular cartilage throughout life in the intensely loaded mechanical environment found in diarthrodial joints. The ability of chondrocytes to regulate their own metabolic activity in response to the mechanical environment enables the chondrocytes to modulate the tissue's structure and composition, and hence, the mechanical properties of the extracellular matrix to the physical demands of the body.

Cartilage explants and chondrocytes have been exposed in vitro to direct mechanical load, hydrostatic pressure and stretching forces (1-3). In vivo, articular cartilage is loaded by external loads, body weight and muscle forces, and as a result, cartilage deforms. Several loading devices have been described to apply direct mechanical forces to cartilage explants. These studies found that static compressive loading resulted in decreased matrix biosynthesis (4-7) whereas an increase (5,6,8-12), decrease (5-7,10,13) or no effect (6,8) was reported after cyclic loading. These inconsistent results can in part be attributed to differences in explants tested, such as immature cartilage (1,10,14) versus mature cartilage (5,7,8,11-13) or articular cartilage with (9,11) or without (5,7,8,10,12,13) the underlying bone. Furthermore, the type of mechanical system used, the radiolabeling period chosen during (6,7,9-13) or after (5,6,8) tissue loading, and chondrocyte viability, which was not determined in any of these studies, may account for the observed discrepancies. For instance, the use of a non-porous load platen (5,6,8,9,11), which effectively seals the articular surface, inhibits the major pathway for nutrients into the tissue and metabolic product transport out of the tissue. In addition, the overall time for tissue loading in most of these previous studies ranged from only hours to a maximum of one day (5-8,12). Also, the distribution of newly synthesized PGs as well as the endogenous PGs in the tissue and medium, has not been extensively described (5,7-10,12,13).

One of our long-term objectives is to manipulate initially disease-free articular cartilage explants with mechanical factors in an attempt to mimic a degenerative metabolism and morphology similar to that seen in osteoarthritic cartilage. In this line, the present study was performed in order to determine systematically the effect of load magnitude, frequency and load duration of a continuously applied, cyclic mechanical load on the biosynthesis and release of proteoglycans (PGs), and the viability of mature articular cartilage explants using our recently reported novel mechanical loading apparatus (15).

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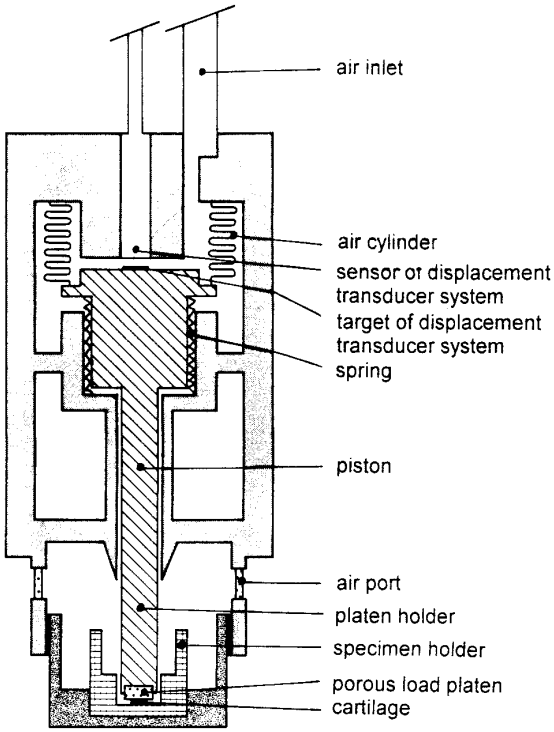

FIG. 1. Schematic drawing of the loading chamber. The cartilage explant is mechanically loaded using a porous load platen connected to a load platen holder and an air cylinder. Eight vents are incorporated to allow exchange with the incubator air under sterile conditions.

MATERIALS AND METHODS

Mechanical loading apparatus. A novel mechanical loading apparatus was used to load cartilage explants over extended periods of time as has been already described in detail (Fig. 1) (15). Briefly, the apparatus consists out of a computerized control unit positioned outside the CO_2-incubator and a mechanical device with six air powered loading chambers designed small enough to fit within a normal bench top incubator. Eight vents are incorporated into the upper part of the chambers to allow air exchange under sterile conditions. The loading chamber consists of two sections. An upper section contains the pneumatic cylinder connected to a load platen holder, and a lower section houses a removable specimen holder, which is made of surgical grade titanium to ensure biocompatibility and to avoid corrosion. The movement of explants within the bottom of the specimen holder is restricted to a 10-mm-diameter area by a toothed ring. The 10-mm-diameter porous load platen is made of glass to ensure tissue biocompatibility, and has a maximum porosity of 40–100 μm. Through a servo-controlled, electropneumatic proportional valve, air is pumped into the stainless steel air cylinder causing the load platen to compress the cartilage.

A displacement transducer system measures the deformation of the articular cartilage with a precision of $\pm$ 10 μm, which is recorded by the data acquisition system of the computerized control unit.

Cyclic loading of cartilage explants. Articular cartilage explants of the weight-bearing area of healthy fetlock joints from 18 to 24 month old steers were removed from the underlying bone using a scalpel. Seven-mm-diameter cartilage explants were then obtained using a biopsy punch. The thickness of each explant was determined twice in the center of the cartilage using a digital caliper with a resolution of 0.01 mm and an accuracy of $\pm$ 0.02 mm. Cartilage explants were placed into the specimen holders with the articular surface upright and were cultured in 2.5 ml serum-free Ham's F-12 nutrient media, pH 7.2, as previously described (16). This medium was further supplemented with 1.0 mM Na_2SO_4 (17), 2.5 μg/ml amphotericin B and 50 μg/ml gentamycin. The loading device was positioned into a CO_2-incubator (model 3039, Forma Scientific, Marietta, U.S.A.) for 2 h to allow equilibration with the incubators environment (37°C, 5% CO_2 and 95% humidity). Continuously applied, uniaxial cyclic loading was applied by using a sinusoidal waveform of 0.001, 0.01, 0.1 or 0.5 Hz-frequency and a peak stress of 0.1, 1.0, 2.5 or 5.0 MPa for a period of 1, 3 or 6 days. In experiments in which cartilage explants were cultured for 6 days, media were changed on day 3. Explants were loaded perpendicular to their long axis in radially-unconfined compression. Collected media was stored frozen at −20°C in the presence of a 10% (v/v) protease inhibitors mixture containing 0.1 mM PMSF, 200 mM EDTA, 5 mM benzamidine/HCl and 10 mM N-ethylmaleimide. During the last 18 h of the experiments, cartilage explants were radiolabeled with 10 μCi/ml $[^{35}S]O_4$. Unloaded cartilage discs from the same condyle were cultured in identically constructed loading chambers, and served as controls.

PG synthesis. At the end of the radiolabeling period, media were harvested and stored frozen at −20°C in the presence of the 10% volume protease inhibitors mixture, until analyzed. The PGs in the load platen were extracted on a rocker for 48 h at 4°C with 1 ml of 4 M guanidinium chloride in extraction buffer containing 50 mM acetate buffer, pH 5.8, 100 mM 6-aminocaproic acid, 5 mM benzamidine/HCl, 10 mM EDTA, 10 mM N-ethylmaleimide and 1 mM PMSF (18), and stored frozen at −20°C until analyzed. Cartilage explants were washed three times with GBSS and stored frozen at −20°C in GBSS together with the 10% (v/v) protease inhibitors mixture until further analysis. Cartilage explants were digested for 4 h at 65°C with 1 ml of 0.5 mg/ml papain digestion solution at a pH 6.5 containing 50 mM monosodium phosphate, 2 mM N-acetylcysteine and 10 mM EDTA. $[^{35}S]O_4$-labeled PGs within the papain digested explants, media and load platen extract were determined by separation of free $[^{35}S]O_4$ from macromolecular $[^{35}S]O_4$-labeled PGs by size exclusion chromatography on Sephadex G-25 columns (Pharmacia, Freiburg, Germany). The sum of $[^{35}S]$-labeled PGs found in the tissue, medium and load platen extract was calculated for determination of the total PG biosynthesis.

Quantitation of GAGs and DNA. Papain digested cartilage explants, culture media, and load platen extracts (25 μl portions) were assayed for sulfated PGs by the reaction with 0.25 ml 1,9-dimethylmethylene blue dye solution in polystyrene 96 well plates and quantitation with spectrophotometry at 523 nm using an ELISA-plate reader. Chondroitin sulfate A from bovine trachea (Sigma GmbH, Deisenhofen, Germany) was used as the standard (19). This method determines endogenous as well as newly synthesized PGs. However, the majority of PGs quantitated by this procedure represent endogenous PGs. The DNA contents of papain digested cartilage explants were determined fluorometrically using the bisbenzimidazol dye Hoechst 33258 as previously described in detail (20).

Chondrocyte viability. As a biochemical indicator of cell viability, the activity of the cytoplasmatic enzyme lactate dehydrogenase (LDH; EC 1.1.1.27) in the culture media was measured using an optimized LDH test (Sigma, Deisenhofen, Germany).

Statistical evaluation. All experiments were repeated 6 times using different specimens. The data obtained from loaded explants were normalized by values from unloaded controls and subsequently analyzed with a one-way analysis of variance (ANOVA) with Tukey's method to compare means. In addition, Spearman nonparametric correlation analysis was performed to obtain the correlation coeficient r. Significance was set $P \leq 0.05$.

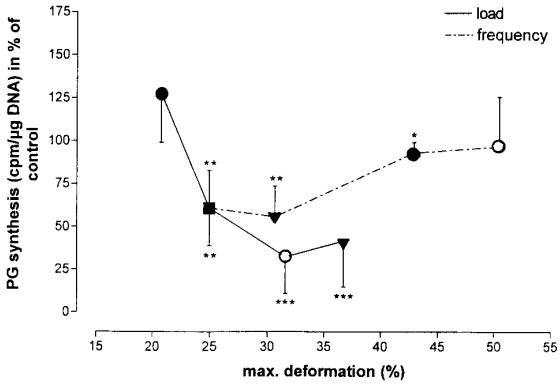

FIG. 2. Effect of load magnitude and frequency on the incorporation of [^{35}S]O_4 into PGs per μg DNA normalized by values obtained from unloaded control cultures. A maximum stress of 0.1 MPa (●), 1.0 MPa (■), 2.5 MPa (○) and 5.0 MPa (▼) was cyclically applied on cartilage discs for 3 days using a 0.1 Hz-frequency (—). In order to determine the effect of frequency on PG synthesis (---), a maximum stress of 1.0 MPa was cyclically applied on cartilage discs for 3 days using a frequency of 0.001 Hz (○), 0.01 Hz (●), 0.1 Hz (■) and 0.5 Hz (▼). Data are mean-values ± S.D. (N=6). Statistically significant difference from unloaded control values: * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

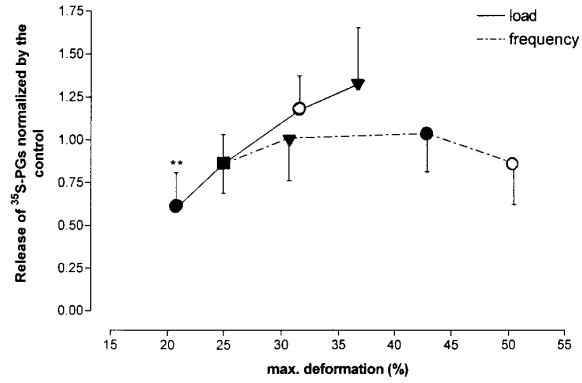

FIG. 3. Effect of load magnitude and frequency on the release of newly synthesized PGs from explants into the nutrient media. [^{35}S]-labeled PGs found in the media per μg DNA was divided by the amount of [^{35}S]-labeled PGs per μg DNA found within the explant. These values were then normalized by data obtained from the corresponding unloaded control cultures. A maximum stress of 0.1 MPa (●), 1.0 MPa (■), 2.5 MPa (○) and 5.0 MPa (▼) was cyclically applied on cartilage discs for 3 days using a 0.1 Hz-frequency (—). In order to determine the effect of frequency on PG synthesis (---), a maximum pressure of 1.0 MPa was cyclically applied on cartilage discs for 3 days using a frequency of 0.001 Hz (○), 0.01 Hz (●), 0.1 Hz (■) and 0.5 Hz (▼). Data are mean-values ± S.D. (N=6). Statistical significant different from unloaded control values: ** $0.001 < P \leq 0.01$.

RESULTS

Cyclic mechanical loading of articular cartilage explants resulted in a load dependent inhibition of PG biosynthesis compared with unloaded control cultures (Fig. 2; $r = -0.84$ with $P = 0.04$). Accumulation of newly synthesized PGs within the nutrient media was increased by elevating the maximum stress from 0.1 to 5.0 MPa on explants (Fig. 3; $r = 0.99$ with $P = 0.009$). Independent from the applied load magnitude, loaded cartilage explants released significantly less endogenous PGs into the nutrient media than unloaded control discs (Fig. 4), whereas the total PG content was unchanged.

Reducing the frequency of loading from 0.5 to 0.001 Hz reduced the inhibition of PG biosynthesis (Fig. 2; $r = 0.93$ with $P = 0.03$) even though explants were more compressed (Fig. 2,6). The total PG content of the cartilage explant cultures remained unchanged. Fig. 3 and 4 demonstrate that reducing the frequency did not affect the release of endogenous PGs ($r = 0.90$ with $P = 0.1$) as well as of newly synthesized PGs ($r = 0.02$ with $P = 0.49$), eventhough explants were more compressed.

Fig. 5 shows that increasing the duration of loading from 1 to 3 or 6 days inhibited the PG biosynthesis of cartilage explants loaded with a maximum stress of 1.0 MPa, which was cyclically applied with a 0.1 Hz-frequency ($r = -0.97$ with $P = 0.01$). The total PG content of cartilage explant cultures remained unchanged. Fig. 5 also demonstrates that an increased load duration inhibited the loss of endogenous PGs from explants ($r = -0.97$ with $P = 0.01$), whereas the release of newly synthesized PGs into the nutrient media was not influenced ($r = 0.78$ with $P = 0.10$).

No activity of LDH in the nutrient media from loaded

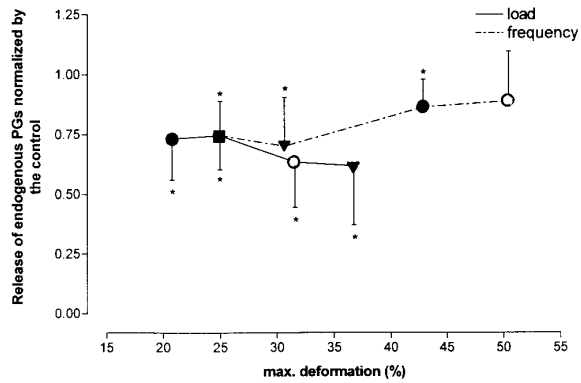

FIG. 4. Effect of load magnitude and frequency on the release of endogenous PGs from explants into the nutrient media as determined by spectrophotometry. The amount of endogenous PGs found in the media per μg DNA was divided by the amount of endogenous PGs per μg DNA found within the explant. These values were then normalized by data obtained from the corresponding unloaded control cultures. A maximum stress ranging from 0.1 MPa (●), 1.0 MPa (■), 2.5 MPa (○) and 5.0 MPa (▼) was cyclically applied on cartilage discs for 3 days using a 0.1 Hz-frequency (—). In order to determine the effect of frequency on PG synthesis (---), a maximum stress of 1.0 MPa was cyclically applied on cartilage discs for 3 days using a frequency of 0.001 Hz (○), 0.01 Hz (●), 0.1 Hz (■) and 0.5 Hz (▼). Data are mean-values ± S.D. (N=6). Statistically significant difference from unloaded control values: * $0.01 < P \leq 0.05$.

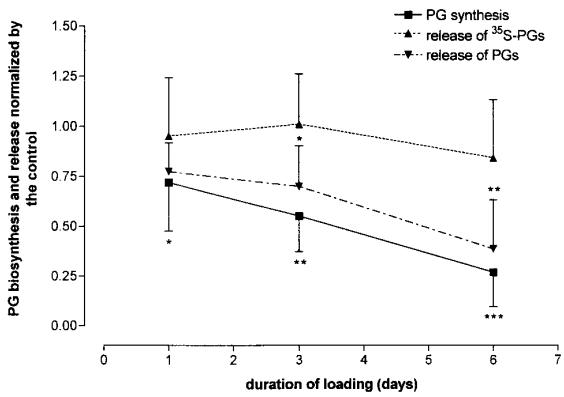

FIG. 5. Effect of load duration on $[^{35}S]O_4$ incorporation into PGs (—) and release of newly synthesized and endogenous PGs from explants into the nutrient media (dotted lines). A maximum stress of 1.0 MPa was cyclically applied on cartilage discs with a frequency of 0.1 Hz. The sum of $[^{35}S]$-labeled PGs found in the explant, media and load platen were normalized by values obtained from the corresponding unloaded control cultures. The amount of newly synthesized or endogenous PGs found in the media and load platen per μg DNA was divided by the amount of endogenous PGs per μg DNA found within the explant. These values were then normalized by data obtained from the corresponding unloaded control cultures. Data are mean-values ± S.D. (N=6). Statistically significant difference from unloaded control values: * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

and unloaded explants cultured for 1 or 3 days could be detected. Preliminary experiments showed that the media of articular cartilage explants, which were killed by 3 freeze-thawing cycles, contained LDH at an enzymatic activity of 30 ± 6.8 U/L per mg wet weight of cartilage explant (N=6). The freeze-thawing cycles did not influence the enzymatic activity of LDH. Cartilage explants loaded for 6 days with a maximum stress of 1.0 MPa, which was cyclically applied with a 0.1 Hz-frequency, released LDH at an activity of 4.1 ± 1.3 U/L per mg wet weight of cartilage.

As determined after 8 h in culture, the degree of compression of loaded explants increased non-linearely by elevating the maximum stress on cartilage from 0.1 MPa to 5.0 MPa (Fig. 6; ANOVA: $p = 0.0001$). Reducing the frequency of loading from 0.5 Hz to 0.001 Hz elevated the maximum deformation of cartilage explants (ANOVA: $p < 0.0001$). During the first 4 minutes of loading, the maximum strain markedly increased after which time the degree of compression remains nearly constant. The standard deviations of mean compression shown in Fig. 6 are lying in the range of ± 5% indicating that loaded cartilage explants, being initially 0.57 ± 0.08 mm (N=60) thick, are compressed to a similar degree.

DISCUSSION

Our study shows that increasing the load magnitude, frequency and duration of loading resulted in a significant inhibition of the PG biosynthesis, and that reducing the frequency decreased the inhibitory effect of a given cyclic stress on PG synthesis. Increasing the load magnitude significantly enhanced the release of newly synthesized PGs from explants into the nutrient media, whereas the release of endogenous PG was significantly reduced by prolonging the duration of loading. In experiments lasting for 1 or 3 days, explants remained viable. Approximately 14 % less living chondrocytes were estimated to be within explants loaded for 6 days compared to unloaded control explants indicating mechanically induced cell death. This may account, however only in part, for the decreased PG biosynthesis in explants loaded for 6 days compared with explants loaded for 1 or 3 days.

The stresses acting in the bovine knee joint in vivo are unknown. Simon (14) reported that the stresses on the cartilage surface in species of widely different sizes are relatively constant. The stresses used in our experiments ranged from 0.1 to 5.0 MPa, which correspond to those found in the human knee joint during walking (0.8-6.3 MPa) (21). Increasing the duration of loading from 1 to 3 or 6 days resulted in a decreased PG synthesis. This effect was only seen in cartilage explants loaded with 1.0 MPa which was applied with a 0.5 Hz-frequency. Reducing the frequency of loading to 0.1 Hz decreased the effect of load duration on PG synthesis, which is in agreement with our other data showing that reducing the load frequency decreased the effect of

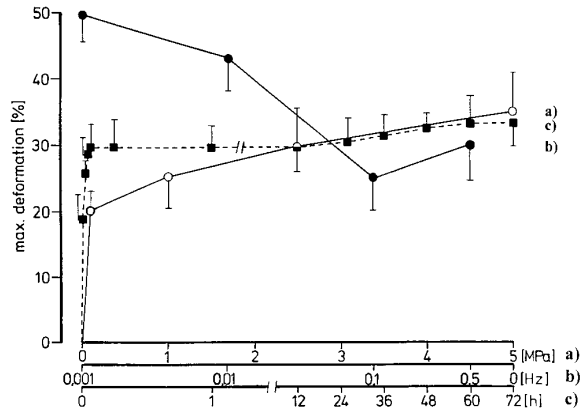

FIG. 6. The percentage of maximum deformation of explants as a function of maximum stress, frequency, and duration of loading. (a) Explant were cyclically loaded with the indicated maximum stress at a frequency of 0.1 Hz (open circles). The effect of loading on the maximum deformation of explants was determined after 8 h in cultures. (b) The effect of loading frequency on the maximum compression of explants was determined after 8 h in cultures, superimposed with a maximum stress of 1.0 MPa (closed circles). (c) The maximum compression was also measured continuously over a period of 3 days in cartilage explants superimposed with a maximum stress of 2.5 MPa, which was cyclically applied with a 0.1 Hz-frequency (dotted line). Values are mean-values ± S.D. (N=6) and are significantly compressed with $p \leq 0.01$.

a given load magnitude on PG synthesis. These results suggest that the load frequency is one of the key determinants of the biosynthetic activity of chondrocytes. Also Sah et al. (6) demonstrated this frequency-dependence of the PG synthesis. These investigators found that in explants subjected to an applied constant cyclical strain, low-frequency oscillations (<0.001 Hz) had no effect on PG biosynthesis while higher frequencies (0.01-1.0 Hz) produced an increase (6).

Sometimes, our results conflict with those reported by others concerning the effect of cyclic loading on the PG synthesis, where an increase (5,6,8-12), a decrease (5-7,10,13), and no changes (6,8) were found. The differences in explants as well as the loading conditions may account for the differences between our results and those of others. In our study, cartilage explants were positioned upright with the articular surface against a porous load platen and, unlike cartilage-bone explants, the partial thickness, immature articular or epiphyseal cartilage used in other studies (6,9,10), full-thickness mature bovine articular cartilage explants were used in our study. While most other investigators are using nonporous platen to load explants (5,6,8,9,11,12), a porous load platen was used in our experiments to allow solute transport through the articular surface. Furthermore, in our study, cartilage explants were loaded for several days with a permanently applied cyclic compressive stress rather than for hours with an intermittently applied cyclic load (5,8-10,12) or a cyclic compressive strain (6). In our experiments, the cyclically compressed explants were unable to reswell to their original volume. Thus a cyclic compression was always superimposed on an already compressed explant. This might explain the close agreement between our reported inhibition of PG biosynthesis, observed in experiments in which the load was applied with high frequencies, with those reported after static loading (4,6,7,13,18).

The results described here show that cyclic mechanical compression can markedly alter the loss of PGs from cartilage explants into the nutrient media. Increasing the load magnitude significantly enhanced the release of newly synthesized PGs from explants into nutrient media. However, a decreased loss of endogenous PGs from loaded explants compared to unloaded control cultures was almost always observed. Loss of matrix components may be triggered by direct physical, chemical or cell mediated mechanisms. Several investigators have reported that PGs lost into the medium are more polydisperse and of smaller average size than PGs remaining in the tissue, and, in addition, are predominantely unable to form macromolecular aggregates (22). This preferential loss of nonaggregating PGs over aggregating PGs is in accord with more rapid diffusion of smaller macromolecules within cartilage (23). In our study, the increased loss of newly synthesized PGs appears to be independent of possible cellular release or activation of proteinases, since Sah et al. (6) reported, that cyclic compression also stimulated matrix loss from biosynthetically inactive cartilage discs bathed in the presence of proteinase inhibitors. These investigators also found that the ^{35}S-labeled PGs lost during cyclic compression were of smaller average size than those from controls, and that they contained a similarly low proportion of about 15 % that could form aggregates in the presence of excess hyaluronate and link proteins. Therefore, the patterns of PG loss, as seen in our experiments, seem to be dependent on the size of PGs and may be therefore attributed to mechanically induced alterations of physical transport phenomena such as convective fluid flow, tissue permeability, and diffusion.

Articular cartilage has been well described as a biphasic material with non-linear viscoelastic properties consisting of a solid phase (extracellular matrix) and a fluid phase (interstitial water) (1). Our experiments, in which the deformation of cultured cartilage explants was determined, confirm the results obtained by other investigators (24) that the degree of deformation is a function of not only the load magnitude but also of load frequency. In our study, the reduction of the load frequencies from 0.5 Hz to 0.001 Hz resulted in a marked increased deformation of explants. When the loading frequency is low, the deformational response of the tissue matrix can easily adapt to the applied load. Therefore, the solid matrix undergoes a large deformation. In contrast, when the loading frequency is high, the tissue deformation cannot respond as quickly as the loading cycles are applied, causing a high elevation in hydrostatic pressure within the cartilage matrix.

Despite considerable investigations, the exact mechanism, whereby mechanical force is converted to a metabolic response in chondrocytes, remain unclear. It has been proposed that fluid loss as well as a decrease in pH as a result of compressive strain might be one of the mechanisms responsible for the inhibition of PG synthesis (1,6,25,26). Alternatively, the chondrocytes may respond directly to changes in shape via the cytoskeleton, stretch activated or inactivated ion channels, and/or via activating second messenger systems which transduce the mechanical signal into a cellular response (1,25,27-30). The concept, that mechanical stress on articular cartilage, a tissue under pressure in vivo, is an important regulator of chondrocyte metabolic activities, is generally accepted. This study demonstrates that articular cartilage metabolism is directly modulated by physical stimuli in vitro. The load magnitude, frequency and load duration seem to act in concert on the chondrocyte biosynthetic activity, while prolonged compression may be responsible for cell damage. Importantly, the level of the biosynthetic activity

of chondrocytes at a given pressure value appears to be only dependent on the load frequency and the duration of loading and less affected by the degree of cartilage compression.

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