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Cyclic Tensile Stretch Modulates Proteoglycan Production by Intervertebral Disc Annulus Fibrosus Cells Through Production of Nitrite Oxide

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Abstract Degeneration of the intervertebral disc is the main pathophysiological process implicated in low back pain and is a prerequisite to disc herniation. Clinically, mechanical forces are important modulators of the degeneration, but the underlying molecular mechanism is not known and needs investigation to identify the biological target. The aim of this work was to study, at the molecular level, the effects of cyclic tensile stretch (CTS) on the production of proteoglycan by intervertebral disc annulus fibrosus cells since proteoglycans seem to be implicated in the dynamic process of intervertebral disc degeneration. Such cells of rabbit were cultured at high density on plates with a flexible bottom. CTS was applied with use of a pressure-operated instrument to deform the plates. With CTS at 1% elongation (1 Hz frequency), the level of ^{35}S-labeled neosynthesized proteoglycans that accumulated in the cellular pool or were secreted in the culture medium did not change, but at 5% elongation, the level was significantly reduced after 8 h of stimulation (30 and 21%, respectively) and further reduced at 24 h (43 and 41%, respectively). Introducing the protein synthesis inhibitor cycloheximide had no effect on this result. Neither aggrecan and biglycan expression nor proteoglycan physical properties were modified. The level of nitrite oxide production significantly increased by 3.5 times after 8 h of 5% elongation. Introducing the nitric oxide synthase (NOS) inhibitors N^G-methyl-L-arginine or *N*-omega nitro-L-arginine diminished the effects of CTS on the production of nitrite oxide and proteoglycans. By contrast, introducing *N*-iminoethyl-L-lysine (a more specific inhibitor of inductible NOS [iNOS]) had little or no effect. Taken together, these results suggest that cNOS activation seems to be more implicated in the 5% CTS modulation of proteoglycan production than iNOS activation. These results suggest that CTS can help regulate the intervertebral disc matrix by decreasing proteoglycan production through a post-translational regulation involving nitrite oxide. This result could be of interest in the development of local therapeutic strategies aimed at controlling intervertebral disc degeneration. J. Cell. Biochem. 90: 148–157, 2003.

Key words: intervertebral disc; mechanical stretch; annulus fibrosus; nitrite oxide; proteoglycan; chondrocyte

INTRODUCTORY STATEMENT

In developed countries, low back pain is a major health problem and accounts for high medical expenses, absenteeism from work, disability, and handicap [Van Tulder et al., 1995]. In the United-States, low back pain affects between 60 and 80% of the population at some point in their lives [Frymoyer and Cats-Baril, 1991]. Although low back pain constitutes an important public health issue, its pathogenesis has received limited study [Freemont et al.,

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2002]. Some clinical, biochemical, and imaging studies have produced evidence that the intervertebral disc, specifically its degeneration, is the main anatomical structure implicated in the pathogenesis of low back pain [Kang et al., 1997; Lam et al., 2000; Luoma et al., 2000]. Thus, degeneration of the intervertebral disc is a major factor associated with low back pain and is a prerequisite to disc herniation [Kang et al., 1997].

In vivo, mechanical forces are important modulators of intervertebral disc degeneration as observed in cartilage [Urban, 1994; Hutton et al., 1998; Lotz et al., 1998], but the molecular mechanisms implicated are not known. Collagens and proteoglycans are the two main components of the extracellular matrix and participate in the process of intervertebral disc degeneration. However, proteoglycans seem to be more implicated in the dynamic process of intervertebral disc degeneration for multiple reasons. Some evidence indicates that intervertebral disc degeneration begins with a progressive decrease in proteoglycan content leading to dehydration of the disc and modification of the load-bearing ability of this tissue [Buckwalter, 1995; Antoniou et al., 1996; Nerlich et al., 1997]. Evidence exists that intervertebral disc cells are implicated in proteoglycan degradation and synthesis [Poiraudeau et al., 1999; Rannou et al., 2000]. The major proteoglycan of the disc is aggrecan, which forms high molecular weight complexes with hyaluronan to produce a large swelling pressure that is important for the intervertebral disc to respond to mechanical stimulation [Johnstone and Bayliss, 1995]. Aggrecan is thought to play a role in maintaining the collagen network and in collagen fibrillogenesis [Schmidt et al., 1990; Watanabe et al., 1997]. Although the turnover of collagen within the disc is estimated to be very slow (>100 years), that of aggrecan is more rapid, with a half-life of 8–300 days in rabbits [Mankin and Lippiello, 1969]. Because of this relatively rapid turnover, the decreased production of aggrecan could significantly induce intervertebral disc degeneration. Finally, it has been shown than an autosomal recessive mutation in the aggrecan gene leads to intervertebral disc degeneration in mice [Watanabe et al., 1997]. Taken together, these results point out the main role of proteoglycans in intervertebral disc degeneration.

The intervertebral disc is composed of two distinct but interdependent tissues: a gelatinous center, called the nucleus pulposus (NP), and the several surrounding coaxial lamellae, which form the annulus fibrosus (AF). Most of the studies exploring the effects of mechanical forces on intervertebral disc metabolism have focused on the effect of hydrostatic pressure and have shown, with use of human or animal disc explants [Terahata et al., 1994; Ishihara et al., 1996; Handa et al., 1997] or cells [Hutton et al., 1999], a modulation of the production of matrix components (proteoglycans, collagens, matrix metalloproteinases [MMPs], or their inhibitors [tissue inhibitor of metalloproteinases]). In all cases, the metabolic modulations were observed mainly in the NP, which indicates that the NP is more responsive than the AF to hydrostatic pressure. However, Panjabi and White [1990] found that mainly AF cells in vivo are subjected to stretch and shear stresses. In only one study, shear stress has been shown to act synergistically with interleukin-1β (IL-1β) on intracellular calcium concentration in AF cells [Elfervig et al., 2001]. In the present work, we hypothesized that cyclic tensile stretch (CTS) could participate in the modulation of the intervertebral disc matrix degeneration by regulating proteoglycan production by AF cells.

MATERIALS AND METHODS

Cell Culture and Cyclic Stretch Experiments

Rabbit intervertebral disc AF cell culture was performed as previously described [Poiraudeau et al., 1999; Rannou et al., 2000a,b]. In these culture conditions, the phenotype of AF cells is maintained [Poiraudeau et al., 1999; Rannou et al., 2000a]. In all experiments, only confluent first-passage AF cells were used, and the effect of CTS on AF cells was studied by comparing stretched and static control AF cells. Confluent AF cells cultured in six-well plates with flexible bottoms were subjected (stretched cells) or not (static control) to CTS. In some experiments, a non-specific inhibitor of nitric oxide synthases (NOSs), N^G-methyl-L-arginine (L-NMA, 1 mM), or a specific inhibitor of inducible NOS (iNOS), *N*-iminoethyl-L-lysine (L-NIL, 10 μM), or a relative specific inhibitor of constitutive NOS (cNOS), *N*-omega nitro-L-arginine (L-NNA, 5 μM) (Sigma, St Louis, MO), was added during stretching. CTS was applied by use of a Flexercell™ stress unit (Flexercell FX3000,

Flexcell^{TM} Intl., Corp., McKeesport, PA) [Banes et al., 1985]. The cell viability after 24-h exposure to CTS was assessed by DNA fragmentation analysis. The cells were maintained in a Ham F12 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY). The experimental protocol delivered 1 or 5% stretch at a frequency of 1 Hz (0.5 s of elongation, followed by 0.5 s of relaxation) for 0.5–24 h. One and five percent correspond to a strain evaluated on annular collagen type-I fibers from the intervertebral disc subjected to external stress (strain is between 1 and 13%) [Broberg, 1983; Klein et al., 1983; Stokes, 1987; Ebara et al., 1996]. One hertz corresponds to the walk frequency.

Radiolabeling Studies

The incorporation of $^{35}SO_4$ into proteoglycans by stretched and static control AF cells was assessed as previously described [Corvol et al., 1978] in the cellular pool and the corresponding culture medium. After CTS application (0.5–24 h), stretched and static control AF cells were incubated in serum and sulfate-free DMEM (Gibco, Paisley, Scotland) plus 1.5 μCi/ml $Na_2[^{35}SO_4]$ corresponding to an excess sulfate concentration (75 MBq/ml, Amersham, Buckinghamshire, England) for an additional 20 h in the presence or absence of 10 μg/ml cycloheximide (Sigma). This period of 20 h was imposed by the curve of $^{35}SO_4$ incorporation into proteoglycans that reached a plateau after 16 h. Proteoglycans were extracted from the culture media and the cell layer with use of 3 M guanidinium chloride in 0.05 M Tris-HCl (Merck, Darmstadt, Germany), pH 7.4, in the presence of protease inhibitors. Aliquots of the guanidinium extract were then spotted on a set of Whatman 3MM paper. The set was used to quantify the high molecular weight/high-charged sulfated proteoglycan subunits, which were precipitated with 1% cetylpyridinium chloride (CPC) in 0.3 M NaCl (Sigma) according to Antonopoulos et al. [1964] as modified by Larsson and Kuettner [1974]. Scintillation liquid was added to each strip, and were counted with use of a Packard Tricarb β-spectrometer. Each measurement was made in triplicate. For each experiment, results were calculated as mean total cellular pool or medium dpm per well of three similarly treated wells. Results are presented as percentage of the static control (mean ± standard deviation) of three experiments.

The elution profiles of proteoglycans secreted into the culture medium by stretched and static control AF cells were analyzed as previously described [Vittur et al., 1983]. Briefly, proteoglycans from culture media were extracted with 5% CPC in the presence of protease inhibitors. The extracts were lyophilized and dissolved in 0.5 M sodium acetate (pH 6.8) (Calbiochem, La Jolla, CA). Aliquots were applied to a Sepharose 2B (Amersham, Uppsala, Sweden) column. Proteoglycans were eluted in associative conditions. Fractions of each eluate were collected and their radioactivity measured by scintillation counting. The total volume (V_t) of the column was determined with use of free $^{35}SO_4$. The partition coefficient (K_{av}) of ^{35}S-labeled proteoglycans in each fraction was calculated as follows:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e represents the elution volume for each column fraction and V_o the void volume for the column.

Reverse Transcriptase-PCR and Preparation of cDNA Probes for Aggrecan and Biglycan

Total cell RNA was extracted from stretched and static control AF cells according to Chomczynski and Sacchi [1987] and synthesized into cDNA by use of MMLV reverse transcriptase (Promega, Madison, WI). The sequences of the sense primers for aggrecan and biglycan were 5′-CTCACCCCGAGAATCAAATG-3′ and 5′-GATGGCCTGAAGCTCAA-3′, with antisense sequences of 5′-AGGAGGTTTCCGCCGCAGTT-3′ and 5′-GGTTTGTTGAAGAGGCTG-3′, respectively. Products of 744 and 406 bp were predicted for aggrecan and biglycan, respectively. Amplification was carried out on a DNA thermal cycler (Perkin-Elmer, Norwalk, CT). The PCR product was analyzed by electrophoresis on a 1.5% agarose gel and purified with use of a gel extraction kit (Qiagen, Santa Clarita, CA) for ligation. To confirm the identity of the probes, PCR products were isolated from an agarose gel, ligated, and cloned into a pGEM-T cloning vector (Promega) according to the manufacturer's instructions. Plasmids containing the PCR inserts underwent sequencing in an automated DNA sequence analyzer (Perkin-Elmer 273 XL) and were

compared with the known rabbit sequences [Bayne et al., 1995; Boykiw et al., 1998]. cDNA inserts from the plasmids were obtained by gel purification of EcoR1 (Boehringer, Mannheim, Germany) digestion products.

Northern Blotting

The RNA samples (10 μg) were electrophoresed and blotted onto a nylon membrane (Hybond N, Amersham, Les Ulis, France). Prehybridation and hybridation were performed with use of the rapid-buffer kit (Amersham). In addition to aggrecan, biglycan was chosen as a target because of the age of the rabbits. Each cDNA probe of aggrecan and biglycan was labeled with [α^{32} P]-CTP by random primer extension (Megaprime DNA labeling kit, Amersham). After hybridization, membranes were washed sequentially with 2× sodium chloride/sodium citrate buffer (SSC)/0.1% sodium dodecyl sulfate (SDS), 1× SSC/0.1% SDS and exposed to Cronex films (Sterling, Newark, NJ) at −80°C for periods of 4–24 h. The same blots were rehybridized with human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA). The films were scanned and the bands quantified with use of the Studio Scan II (Agfa, Paris, France) and the NIH software supplied. The results are presented as percentage of the static control after normalization of mRNA to GAPDH mRNA.

Nitrite Determination

After CTS application (0.5–24 h), the nitrite levels of the culture medium were determined through indirect measurement of nitric oxide (NO) production by the method of Griess [Evans et al., 1996].

Statistical Analysis

Analysis of variance (ANOVA) was performed with variable duration. When an F value was found to be significant, the ANOVA was followed by multiple comparisons with use of the Tukey test. P values above 0.05 were not considered statistically significant.

RESULTS

Cyclic Tensile Stretch Decreases Proteoglycans Neosynthesized by Annulus Fibrosus Cells

Confluent AF cells cultured on flexible-bottomed culture plates were subjected or not to 1 or 5% CTS for 0.5–24 h. The morphologic features of stretched cells, evaluated under a phase contrast microscope, remained unchanged even after 24 h of stretching. Adherent AF cells have a rounded or polygonal structure, non-significant detachment from the substrate, and no cell death, as assessed by DNA fragmentation were observed [Poiraudeau et al., 1999; Rannou et al., 2000a; data not shown]. CTS applied at 1% elongation did not modify the amount of $^{35}SO_4$ incorporation into neosynthesized proteoglycans in the cellular pool ($F = 0.40$, $P = 0.81$) or in the culture medium ($F = 2.05$, $P = 0.18$) (Fig. 1A) but at 5% elongation induced a significant decrease in the amount of $^{35}SO_4$ incorporation in the cellular pool ($F = 7.90$, $P < 0.001$) and the culture medium ($F = 5.70$, $P = 0.002$) (Fig. 1B). During 0.5–6 h of CTS application, the amount of ^{35}S-labeled proteoglycans remained stable in the cellular pool and in the culture medium, but after 8 h, the amount was significantly reduced in the cellular pool (30%, $P = 0.02$) and the culture medium (21%, $P = 0.05$). This effect was observed until 24 h (down by 43% in the cellular pool and 41% in the culture medium as compared with that in static controls).

Cyclic Tensile Stretch-Induced Decreased Proteoglycan Production Does not Require Protein Synthesis

In order to determine whether the effect of CTS on neosynthesized proteoglycans was direct, $^{35}SO_4$ incorporation into neosynthesized proteoglycan sulfate was studied in the presence of the protein synthesis inhibitor cycloheximide. As shown in Figure 1C, the decreased $^{35}SO_4$ incorporation was still observed whatever the time of incubation ($F = 25.58$, $P < 0.001$; and $F = 21.3$, $P < 0.001$, for the cellular pool and the culture medium, respectively). This indicates that protein synthesis is not required for the CTS effect to occur.

Cyclic Tensile Stretch Does not Modify Aggrecan and Biglycan Gene Expression

To examine further the mechanism implicated in the CTS-induced decrease of neosynthesized proteoglycan production, we studied the mRNA levels of aggrecan and biglycan expressed in AF cells by Northern blotting. One

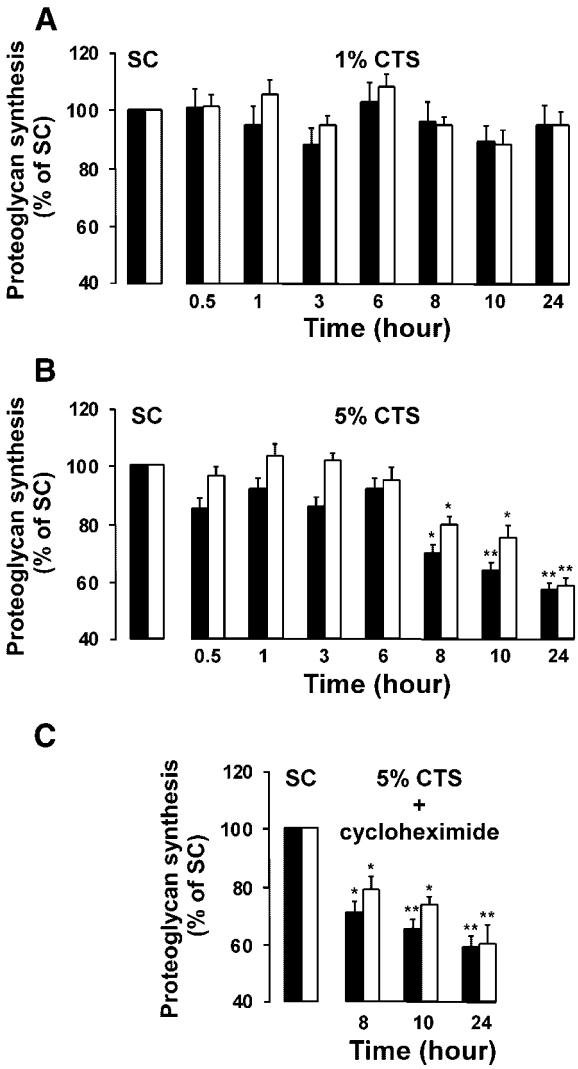

Fig. 1. Effect of cyclic tensile stretch (CTS) on $^{35}SO_4$ incorporation into proteoglycans neosynthesized and secreted by annulus fibrosus (AF) cells in culture. $^{35}SO_4$ incorporation into proteoglycans synthesized in the cellular pool (■) and secreted in the medium (□) by AF cells subjected to 1 or 5% CTS between 0.5 and 24 h was compared with that in static control (SC) AF cells. After CTS application, AF cells were labeled with $^{35}SO_4$ for 20 h in the absence (**A**, **B**) or presence (**C**) of 10 μg/ml cycloheximide. Proteoglycans were extracted with 1% cetylpyridinium chloride in 0.3 M NaCl in the presence of protease inhibitors. Results are presented as percentage of SC (mean ± standard deviation) of three independent experiments. * = $P < 0.05$ relative to SC. ** = $P < 0.01$ relative to SC.

8.9-kb mRNA aggrecan transcript and one 2.1-kb mRNA biglycan transcript were observed under basal conditions (Fig. 2A). CTS applied at 5% for 0.5–24 h had no effect on the ratio of aggrecan to GAPDH or biglycan to GAPDH mRNA when compared with that of static controls (Fig. 2B).

Cyclic Tensile Stretch Does not Modify the Physical Properties of Neosynthesized Proteoglycans

To analyze further the effect of CTS on the physical properties of neosynthesized sulfated proteoglycans secreted into the culture medium, the extent of proteoglycan aggregation was determined after chromatography on Sepharose 2B. More than 90% of ^{35}S-labeled proteoglycans were eluted as large molecular weight complexes ($K_{av} = 0.13–0.15$) under static conditions. CTS had no effect on the elution profile after 0.5-, or 8-, or 24-h application at 1% (Fig. 3A) and 5% elongation (Fig. 3B).

Cyclic Tensile Stretch Increases Nitrite Production

Because NO has often been associated with a modulation of proteoglycan production by chondrocytic cells [Stefanovic-Racic et al., 1996], nitrite production was evaluated in the culture medium of stretched and static control AF cells. The concentration of the three NOS inhibitors used in the present studies came from experiments of NOS activities evaluated on IL-1β-treated rabbit articular chondrocytes for iNOS specificity and rabbit brain homogenate for cNOS specificity. These concentrations inhibited more than 90% of the NOS activities of IL-1β-treated articular chondrocytes and brain homogenate and had no effect on nitrite concentration in static control AF cells (data not shown). The basal production of nitrites observed in the culture medium of AF cells was at the limit of detection and evaluated at a mean of 4.01 ± 0.52 μM after 0.5 h (Fig. 4). Nitrite concentration remained stable for 0.5–24 h in static controls. CTS at 1% elongation had no effect on nitrite basal concentration, but a significant 3.5-fold increase was observed after 8 h at 5% CTS ($F = 27.52$, $P < 0.001$) (Fig. 4). There was no difference in effect between 8 and 24 h of 5% CTS. The 5% CTS-induced nitrite production was totally abolished by the addition of L-NMA and partially abolished by the addition of L-NNA ($P < 0.01$) (Fig. 4). By contrast, the introduction of L-NIL did not abolish the 5% CTS-induced nitrite production after 24 h ($P = 0.8$). After 8 h, we observed a nonsignificant decrease of nitrite production as compared to that at 5% CTS ($P = 0.1$) (Fig. 4). Taken together, these results suggest than cNOS activation is implicated more with the 5%

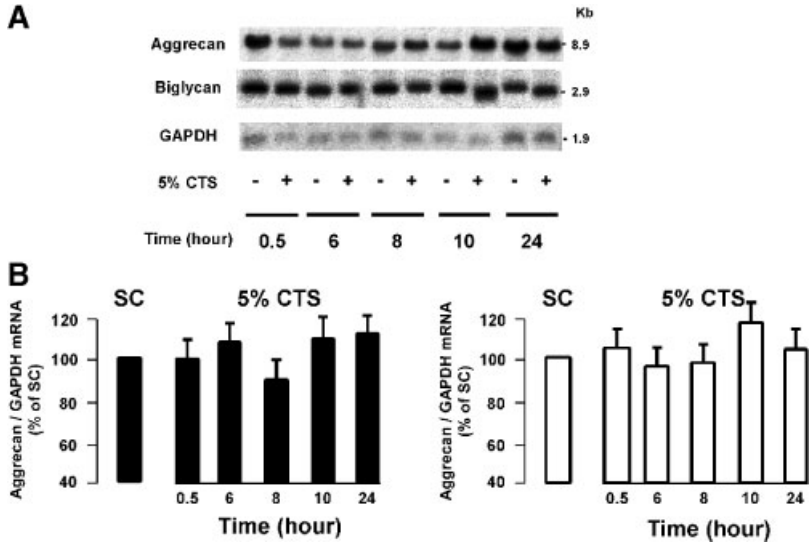

Fig. 2. Effect of CTS on AF aggrecan and biglycan mRNA contents. Northern blotting of aggrecan and biglycan mRNA in AF cells subjected or not to CTS at 5% for 0.5 to 24 h. **A**: Total cell RNA was extracted, and samples (10 μg) were electrophoresed in denatured 2.2 M formaldehyde, 0.5% ethidium bromide, and 1% agarose gels and blotted onto a nylon membrane. The blot was hybridized with ^{32}P-labeled aggrecan and biglycan probes. The amount of RNA loaded in each lane was monitored by hybridization with a human GAPDH cDNA. This Northern blot is from one representative experiment out of three. **B**: Aggrecan (■) and biglycan (□) mRNA expression was normalized to the corresponding GAPDH mRNA, and results are presented as percentage of SC (mean ± standard deviation) of three independent experiments.

CTS-induced nitrite production than iNOS activation for long durations of stretch (24 h).

Nitric Oxide Synthases Inhibitors can Abrogate the Cyclic Tensile Stretch-Dependent Inhibition of Proteoglycans Synthesized and Secreted by Annulus Fibrosus Cells

To study the implication of the CTS-induced NO production on the CTS-induced decreased production of ^{35}S-labeled proteoglycans, cells were incubated with 1 mM L-NMA, 10 μM L-NIL, or 5 μM L-NNA, and $^{35}SO_4$ incorporation into secreted neosynthesized proteoglycans was measured after 8 and 24 h of CTS application at 5% elongation. As shown in Figure 5, CTS-induced decreased production of ^{35}S-labeled proteoglycans was inhibited in part after 8 h and totally inhibited after 24 h in the presence of L-NMA or L-NNA. By contrast, this inhibition was not observed in the presence of the iNOS specific inhibitor L-NIL after 24 h (Fig. 5B). However, after 8 h, the proteoglycan production in presence of L-NIL partially recovered but was lower than that in presence of L-NNA ($P < 0.05$) (Fig. 5A). These results suggest that the CTS-induced inhibition of proteoglycan production requires NO production mainly through cNOS activation for long periods of stretch (24 h). For shorter stretch durations (8 h), both cNOS and iNOS activation seems to be implicated in the CTS-induced inhibition of proteoglycan production.

DISCUSSION

Our results show that CTS can decrease the production of proteoglycans by AF cells and that this phenomenon depends on the amount and duration of stretch. This CTS-induced decreased proteoglycan production does not require protein synthesis. In addition, no modification in mRNA aggrecan and biglycan content is observed, which suggests that CTS could affect intracellular transport and assembly and/or degradation of proteoglycans. The absence of modified aggregative properties of neosynthesized proteoglycans indicates that CTS does not induce proteoglycan degradation. In addition, we have also observed that CTS did not induce cell death or MMP-1 and MMP-3 expression (data not shown). Finally, our results indicate that CTS regulates sulfate incorporation into proteoglycans neosynthesized by intervertebral disc cells through a mechanism dependent on NO production.

According to Kang et al. [1996], degenerated human discs produce spontaneously more NO than do normal discs. In addition, Liu et al.

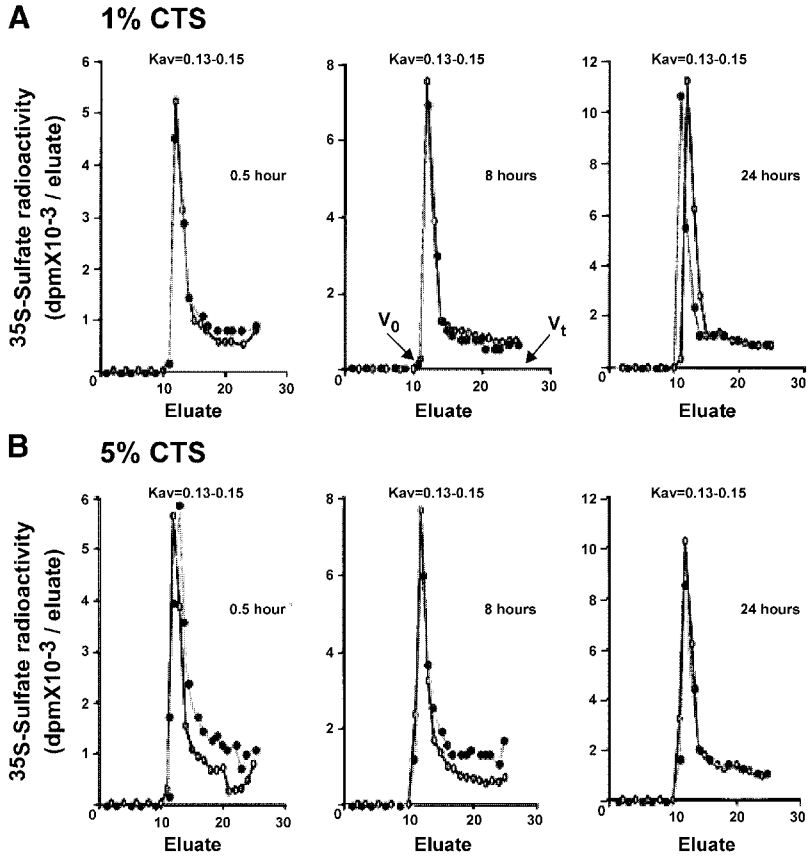

Fig. 3. Effect of CTS on the physical properties of neosynthesized sulfated proteoglycans. Sepharose 2B elution profile of ^{35}S-labeled proteoglycans synthesized by AF cells after 0.5, 8, or 24 h of CTS application at 1 (**A**) and 5% (**B**) as compared with that in static control AF cells. Stretched (●) and static control (○) AF cells were labeled with $^{35}SO_4$ for 20 h. As described in Methods, the proteoglycans were extracted with 5% cetylpyridinium chloride in the presence of protease inhibitors. Aliquots (500 µl) were applied to a column of sepharose 2B and proteoglycans eluted in associative conditions with 0.5 M sodium acetate, pH 6.8. Fractions (100 µl) of each eluate were collected and their radioactivity measured by scintillation counting. The partition coefficient (Kav) of ^{35}S-labeled proteoglycans in each fraction was calculated as follows: $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e represents the elution volume for each column fraction, V_o the void volume for the column, and V_t the total volume of the column. Data are from one representative experiment out of three.

[2001] showed that hydrostatic pressure-induced NO production decreases proteoglycan synthesis in degenerated human lumbar discs. These results point out a close connection between mechanical stress, proteoglycans, and NO in the degeneration process of the discs. However, the exact cellular origin of the increased production of NO in degenerated discs is unknown. Our results suggest that NO can be produced by intervertebral disc cells and not necessarily by cells from infiltrating granulation or vascular tissue. In addition, mechanical stretch could be one of the main factors implicated in autocrine–paracrine NO production and thus of proteoglycan production by AF cells leading to the modulation of intervertebral disc degeneration. Modification of proteoglycan content in the intervertebral disc extracellular matrix can therefore lead to modification of the mechanical properties of this tissue, thus accelerating the degeneration process.

The mechanism implicated in the mechanical modulation of NO production remains unclear. There is no study on this topic that involves AF cells, but this question is debated for articular chondrocytes and fibrochondrocytes of the temporomandibular joint (TMJ). CTS applied to articular chondrocytes and fibrochondrocytes is accompanied by a decrease of NO production, which depends on the downregulation of the *iNOS* gene [Gassner et al., 1999; Agarwal et al., 2001]. This phenomenon was observed only with the presence of IL-1β and for a higher intensity of stimulation (20 and 6%, respectively) than that used in the present study [Gassner et al., 1999; Agarwal et al., 2001]. In

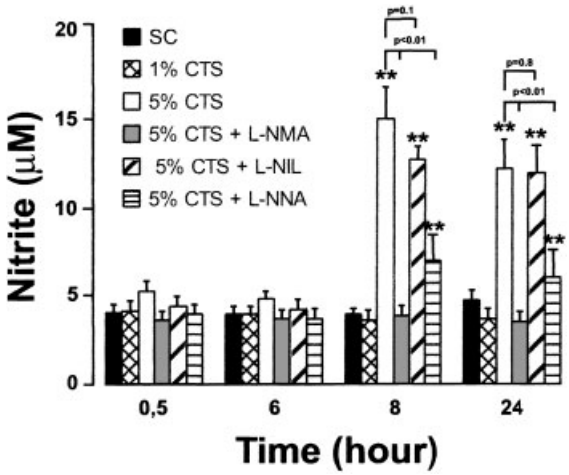

Fig. 4. Effect of CTS on nitrite oxide (NO) production by AF cells. Accumulation of NO in the culture supernatant after 0.5–24 h was assessed by Griess reaction in various conditions: static control AF cells (SC), AF cells subjected to 1% CTS (1% CTS), AF cells subjected to 5% CTS (5% CTS), AF cells subjected to 5% CTS in presence of the NO synthase inhibitor N^G-methyl-L-arginine (L-NMA, 1 mM) (5% CTS + L-NMA), the NO synthase inhibitor *N*-iminoethyl-L-lysine (L-NIL, 10 μM) (5% CTS + L-NIL), and the NO synthase inhibitor *N*-omega nitro-L-arginine (L-NNA, 5 μM) (5% CTS + L-NNA). Results are presented as mean ± standard deviation of three independent experiments. ** = $P < 0.01$ relative to SC.

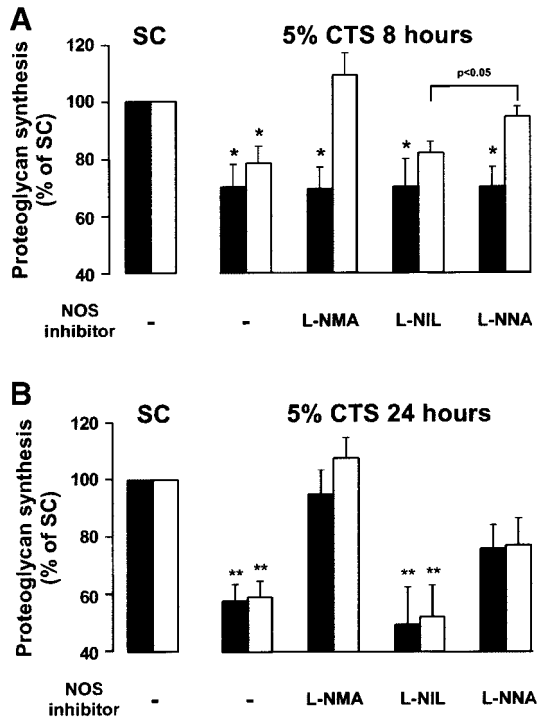

Fig. 5. Effect of nitric oxide synthase (NOS) inhibitors on $^{35}SO_4$ incorporation into proteoglycans neosynthesized and secreted by AF cells subjected to 5% CTS. $^{35}SO_4$ incorporation into proteoglycans synthesized in the cellular pool (■) and secreted in the medium (□) by AF cells subjected to 5% CTS for 8 h (A) or 24 h (B) in the presence or not of the NO synthase inhibitors N^G-methyl-L-arginine (L-NMA, 1 mM), *N*-iminoethyl-L-lysine (L-NIL, 10 μM), or *N*-omega nitro-L-arginine (L-NNA, 5 μM) as compared with that in static control AF cells (SC). AF cells were labeled with $^{35}SO_4$ for 20 h. Proteoglycans were extracted with 1% cetylpyridinium chloride in 0.3 M NaCl in the presence of protease inhibitors. Results are presented as percentage of SC (mean ± standard deviation) of three independent experiments. * = $P < 0.05$ relative to SC. ** = $P < 0.01$ relative to SC.

our work, the production of NO does not require protein synthesis, is not abolished by a specific inhibitor of iNOS (L-NIL) and is partially abolished by a relative specific inhibitor of cNOS (L-NNA), which suggests that probably, modulation of cNOS activity is mainly involved in this observed phenomenon. This observation is in accordance with previous results showing that compressive stress applied to chondrocytes might induce a modulation of NO production through modulation of cNOS activity without transcriptional regulation of NOS genes [Lee et al., 2000]. Taken together, these data emphasize the importance of the cell type and the inflammatory state of the mechanical-stimulated cells.

The intensity of the mechanical stretch able to induce an increase of NO production in AF cells is between 1 and 5% elongation, which suggests that mechanosensors in AF cells can differentiate two low-stretch intensities and respond differently. These mechanosensors are not yet identified but could be the stretch-activated Ca^{2+}-channels. A recent study from Elfervig et al. [2001] has shown than Ca^{2+} concentration in human AF cells is increased three times by mechanical stress as compared to static controls. This effect was observed only in presence of extracellular Ca^{2+}, which indicates a Ca^{2+} flow-through plasma membrane and suggests the involvement of stretch-activated Ca^{2+}-channels in the signaling pathway of mechanical stress. With these observations, together with our results, we could hypothesize that in AF cells, mechanical stress via stretch-activated Ca^{2+}-channels modulates a Ca^{2+}-dependant enzyme like cNOS but not iNOS, which is known to be Ca^{2+}-independant.

How NO modulates proteoglycan production by AF cells subjected to mechanical stress requires further investigation. Contradictory results have been published concerning the chondrocyte. Several reports support the hypothesis that NO only influences cell proliferation and may not be directly implicated in the

production of proteoglycans [Stefanovic-Racic et al., 1996; Lee et al., 2000]. However, mechanical stimulation has been shown to decrease $^{35}SO_4$ incorporation into neosynthesized proteoglycans in the presence of IL-1β [Xu et al., 2000], and this effect was dependent on NO production. Our results confirm that NO is implicated in decreased proteoglycan production and that this effect is not the result of decreased protein synthesis or increased proteoglycan degradation. One hypothesis could be that NO directly inhibits glycosaminoglycan sulfatation, the last step of aggrecan intracellular synthesis, as suggested by Hickery and Bayliss [1998]. The other hypothesis could be that NO has not a direct effect on proteoglycan production but acts more like a second messenger in the mechanotransduction process, as is seen in other cell types.

In conclusion, CTS can participate in the regulation of the intervertebral disc matrix by decreasing proteoglycan production through NO regulation. This regulation seems to be related to NOS activation. This result could be of interest in the development of local therapeutic strategies aimed at controlling intervertebral disc degeneration.

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