Effects of anti-arthritis preparations on gene expression and enzyme activity of cyclooxygenase-2 in cultured equine chondrocytes

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Objective—To determine the effects of recombinant equine interleukin -1β (reIL-1β) and 4 anti-inflammatory compounds on the expression and activity of cyclooxygenase (COX)-2 in cultured equine chondrocytes.

Sample Population—Articular cartilage from 9 young adult horses.

Procedure—Reverse transcriptase-polymerase chain reaction methods were used to amplify a portion of equine COX-2 to prepare a cDNA probe. Northern blot analysis was used to quantify the expression of COX-2 in first-passage cultures of equine articular chondrocytes propagated in media containing dexamethasone (DEX), phenylbutazone (PBZ), polysulfated glycosaminoglycan, and hyaluronan, each at concentrations of 10 and 100 µg/ml and each with or without reIL-1β. A commercial immunoassay was used to determine prostaglandin E_2 (PGE_2) concentrations in conditioned medium of similarly treated cells to quantify COX-2 activity.

Results—Addition of reIL-1β increased the expression of COX-2 in a dose-dependent manner, which was paralleled by an increased concentration of PGE_2 in culture medium. Concentration of PGE_2 in spent medium from reIL-1β-treated chondrocytes was significantly reduced by DEX and PBZ; however, only DEX significantly reduced gene expression of COX-2.

Conclusions and Clinical Relevance—Prostaglandin E_2 is considered to be an important mediator in the pathophysiologic processes of arthritis, and cultured chondrocytes respond to interleukin-1 with enhanced expression and activity of COX-2. Palliative relief in affected horses is probably attributable, in part, to inhibition of PGE_2 synthesis; however, analysis of these data suggests that of the 4 compounds tested, only DEX affects pretranslational regulation of the COX-2 gene in cultured equine chondrocytes. (*Am J Vet Res* 2002;63:1134–1139)

Prostaglandins (PG) are produced from cell membrane phospholipids by the sequential actions of a series of enzymes collectively termed the arachidonic acid cascade. An important and much-studied member of the cascade is **cyclooxygenase (COX)**, the first of a series of enzymes that convert arachidonic acid to PG. Cyclooxygenase represents an important step in regulation of the PG synthetic pathway, and inhibition of the activity of this enzyme is an important mechanism of action for corticosteroids and **nonsteroidal anti-inflammatory drugs (NSAID)**.

Two forms of COX exist. The first (COX-1) produces physiologic concentrations of PG in a constitutive manner. The second (COX-2) is an inducible form of the enzyme that is responsible for increased amounts of PG observed during inflammatory events.[1,2] Inflammatory mediators such as **interleukin (IL)**-1 and lipopolysaccharide increase expression and activity of COX-2 in a number of tissues.[1-5]

Prostaglandin E_2 (PGE_2) is the most abundant prostanoid found in diseased joints, and although the specific effects of this molecule on joint metabolism are unclear, it is widely believed that PGE_2 contributes to lesions of osteoarthritis. Synovial membranes and articular cartilage are capable of synthesizing PGE_2, and it is found in increased concentrations in diseased joints, where it has been linked to synovial inflammation, bone erosion, and cartilage matrix depletion.[6-8] Indeed, it has been suggested that PG modulate the release of cartilage-degrading neutral metalloproteinases such as collagenases and stromelysins, enzymes considered pivotal in the degeneration of cartilage matrix during osteoarthritis.[9,10] Conversely, there is increasing evidence to suggest that matrix metalloproteinase expression in articular cells is inhibited by E-series PG,[11] data that are supported by experiments that used equine tissues.[12] These and other data suggest that PGE_2 may serve a regulatory function in inflamed joints, and inhibition of their synthesis may not produce uniformly favorable results.[13,14] Thus, establishing the true role of PG in osteoarthritis is critical, in part, because NSAID receive such widespread use for treatment of animals affected with the disease, and their indiscriminate use can have profound systemic adverse effects in addition to potential direct and indirect effects on health of cartilage matrix.

Various compounds are commonly used in palliative treatment of horses with osteoarthritis, including corticosteroids, NSAID, and putative disease-modifying (chondroprotective) agents such as hyaluronan and **polysulfated glycosaminoglycan (PSGAG)**. Although such compounds are often effective in reducing lame-

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ness and possess at least some cartilage-sparing potential, the specific mechanisms by which these and other medications provide relief and retard the progression of osteoarthritic lesions remain unclear. A potential mechanism of action of some or all of these agents may include modification of expression or activity of COX-2. To address this issue, the study reported here was conducted to determine the effects of **recombinant equine IL-1β (reIL-1β)** and a number of anti-inflammatory compounds on the expression and activity of COX-2 in cultured equine chondrocytes.

Materials and Methods

Source of tissues and cell culture—Macroscopically normal articular cartilage was obtained from the metacarpophalangeal and metatarsophalangeal joints of 9 young adult (2 to 8 years old) horses, and chondrocytes were liberated by sequential digestion with pronase and collagenase as described elsewhere.[15] Briefly, cartilage slices were incubated at 37 C with filter-sterilized solutions of pronase (1.0 mg/ml for 1 hour) and collagenase (0.5 mg/ml for 18 hours). Following centrifugation and washing, high-density monolayer cultures were established in Dulbecco modified Eagle medium with 10% fetal calf serum and antibiotics by use of standard culture conditions. Once confluent, cells were treated with trypsin and plated at high density in 6-well plates for specific experiments. Cells were confluent within 36 hours and were deprived of serum for 3 to 5 days prior to the start of experiments to reduce their metabolic activity.

Amplification of cDNA and probe preparation—The cDNA was synthesized by use of a **reverse transcriptase-polymerase chain reaction (RT-PCR)** method that used 2 µg of total RNA from a chondrocyte culture that had been stimulated for 8 hours with 10 ng of reIL-1β/ml. A specific set of oligonucleotide primers for COX-2 was synthesized on the basis of the corresponding published human cDNA sequences and was used at a final concentration of 3 p*M*/µl. The primer sequences were 5'-ATT AAT GCC AGC GCT TCC CAC TC-3' for the sense primer and 5'-ACC TAC AAA CCC ATG CCA ACT GA-3' for the antisense primer. Amplification by use of the PCR procedure produced a 776-**base pair (bp)** cDNA fragment for COX-2, which was ligated directly to a pCR-2.1 vector.[a] Sequencing of the PCR product was performed by use of an automated sequencer.

Time course and dose-response—To characterize temporal aspects of COX-2 expression, first-passage chondrocytes from grossly normal articular cartilage were grown to confluence in 6-well plates and exposed to reIL-1β (10 ng/ml) for 0, 6, 12, 24, 48, and 72 hours, which was followed by isolation of RNA and northern blot hybridization. To establish a subsaturating dose of reIL-1β for COX-2 expression that could be used in subsequent experiments, similar cultures were stimulated with 0, 1, 5, 10, 50, and 100 ng of reIL-1β/ml for 6 hours followed by harvesting of total RNA for northern blot analysis and quantification of relative expression.

Treatment of stimulated chondrocytes with anti-arthritis preparations—First-passage chondrocytes derived from grossly normal articular cartilage obtained from the metacarpophalangeal and metatarsophalangeal joints of 4 horses were incubated in three 6-well plates. After a period of 3 to 5 days of culture in serum-free conditions, each well received a randomly assigned treatment. Treatments consisted of **dexamethasone (DEX**; (10^{-6} and 10^{-5}*M*), **hyaluronan (HA**; 10 and 100 µg/ml), PSGAG (10 and 100 µg/ml), or **phenylbutazone (PBZ**; 10 and 100 µg/ml) with and without the addition of reIL-1β (5 ng/ml). In addition, there was a control treatment (no additives) and a treatment that consisted of 5 ng of reIL-1β without anti-arthritis agents. After incubation for 6 hours, RNA was extracted for northern blot analysis. Conditioned medium from identically prepared cultures of tissues obtained from joints of 3 horses was incubated for an additional 24 hours after treatment and used for determination of PGE_2 concentrations.

Determination of PGE_2 concentration—Concentration of PGE_2 in media samples was determined by use of a commercially available colorimetric assay,[b] which was conducted in accordance with the manufacturer's directions. Briefly, 10-fold dilutions of conditioned media were incubated in an assay buffer containing a fixed amount of alkaline phosphatase-labeled PGE_2 in 96-well plates coated with a murine monoclonal antibody to PGE_2. Following washing, *p*-nitrophenyl phosphate was added as a substrate for alkaline phosphate, and absorbance was measured on a spectrophotometer at a wavelength of 405 nm. Concentrations of PGE_2 were subsequently determined by use of a standard curve generated according to a 4-parameter logistic curve-fitting function.[c]

Northern blot analysis—Northern blot hybridization was conducted by use of conventional methods reported elsewhere,[15] using hybridization solution that contained 100 ng of digoxigenin-labeled COX-2 probe/ml. Detection was accomplished by use of a chemiluminescent method[d] performed in accordance with the manufacturer's instructions. Membranes then were subjected to autoradiography at 25 C for 15 minutes. Autoradiographic films were scanned by use of a desktop scanner, and intensity of each band was quantified by use of image-acquisition and analysis software[e] to

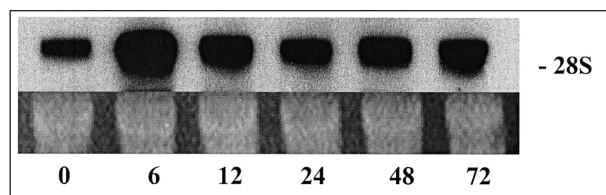

Figure 1—Representative northern blot revealing temporal aspects of up-regulation of expression of cyclooxygenase (COX)-2 by normal equine chondrocytes stimulated with 10 ng of recombinant equine interleukin (reIL)-1β. Total RNA (4 µg) was resolved on formaldehyde agarose gels. Blots were probed with a digoxigenin-labeled COX-2 cDNA probe. Duration of exposure (number of hours) of the cultures to the cytokine prior to RNA isolation is indicated beneath each of the COX-2 bands. The position of the 28S rRNA band with respect to the COX-2 bands is indicated, and ethidium bromide-stained 28S rRNA bands corresponding to each COX-2 band are shown in the bottom panel.

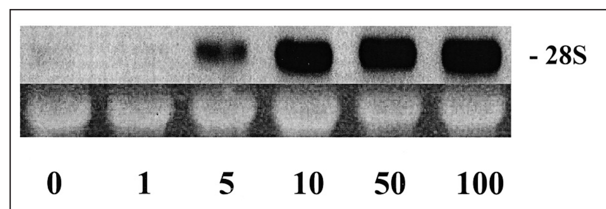

Figure 2—Representative northern blot revealing COX-2 expression by normal equine chondrocytes stimulated with increasing doses of reIL-1β after incubation for 6 hours. Total RNA (4 µg) was resolved on formaldehyde agarose gels. Concentrations of reIL-1β (in nanograms per milliliter) are indicated below each corresponding lane. The position of the 28S rRNA band with respect to the COX-2 bands is indicated, and ethidium bromide-stained 28S rRNA bands corresponding to each COX-2 band are shown in the bottom panel.

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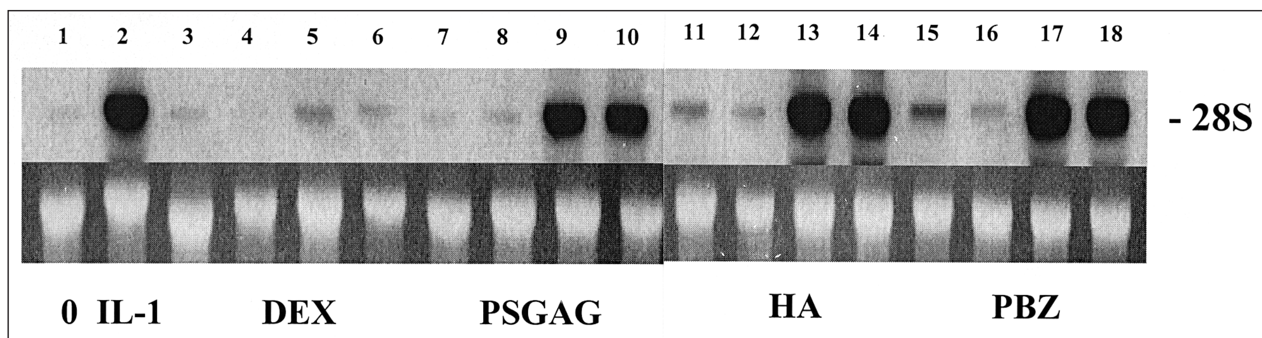

Figure 3—Representative northern blot revealing effects of reIL-1β-(IL-1) stimulated COX-2 expression by normal chondrocytes cultured with various anti-arthritis preparations. Total RNA (4 µg) was resolved on formaldehyde agarose gels. The position of the 28S rRNA band is indicated to the right of the COX-2 bands. The ethidium bromide-stained 28S rRNA bands corresponding to each COX-2 band are shown in the bottom panel. Specific treatments for each lane were as follows: 1, control (medium alone); 2, 5 ng of reIL-1β/ml; 3, $10^{-6}M$ dexamethasone (DEX); 4, $10^{-5}M$ DEX; 5, $10^{-6}M$ DEX plus 5 ng of reIL-1β/ml; 6, $10^{-5}M$ DEX plus 5 ng of reIL-1β/ml; 7, 10 µg of polysulfated glycosaminoglycan (PSGAG); 8, 100 µg of PSGAG; 9, 10 µg of PSGAG plus 5 ng of reIL-1β/ml; 10, 100 µg of PSGAG plus 5 ng of reIL-1β/ml; 11, 10 µg of hyaluronan (HA); 12, 100 µg of HA; 13, 10 µg of HA plus 5 ng of reIL-1β/ml; 14, 100 µg of HA plus 5 ng of reIL-1β/ml; 15, 10 µg of phenylbutazone (PBZ); 16, 100 µg of PBZ; 17, 10 µg of PBZ plus 5 ng of reIL-1β/ml; 18, 100 µg of HA plus 5 ng of reIL-1β/ml.

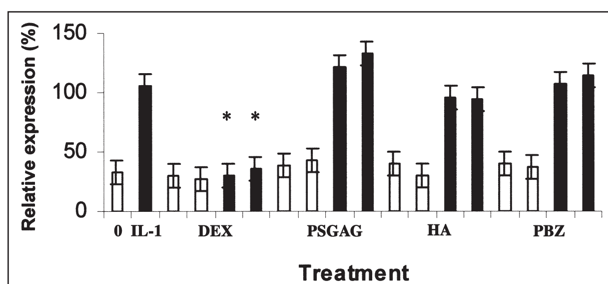

Figure 4—Expression of COX-2 by equine chondrocytes obtained from grossly normal articular cartilage that was cultured with various anti-arthritis preparations without (open bars) and with (solid bars) addition of 5 ng of reIL-1β/ml. The COX-2 expression was calculated as ratio of the intensity of the bands for inducible nitric oxide synthase to intensity of the ethidium bromide-stained 28S ribosomal RNA band of the electrophoretic gel. Values are mean ± SEM from 4 experiments. Treatments were as follows: control (0), medium only; IL-1, 5 ng of reIL-1β/ml; DEX, $10^{-6}M$ alone, $10^{-5}M$ alone, $10^{-6}M$ plus 5 ng of reIL-1β/ml, and $10^{-5}M$ plus 5 ng of reIL-1β/ml; PSGAG, 10 µg of PSGAG alone, 100 µg of PSGAG alone, 10 µg of PSGAG plus 5 ng of reIL-1β/ml, and 100 µg of PSGAG plus 5 ng of reIL-1β/ml; HA, 10 µg of HA alone, 100 µg of HA alone, 10 µg of HA plus 5 ng of reIL-1β/ml, and 100 µg of HA plus 5 ng of reIL-1β/ml; and PBZ, 10 µg of PBZ alone, 100 µg of PBZ alone, 10 µg of PBZ plus 5 ng of reIL-1β/ml, and 100 µg of HA plus 5 ng of reIL-1β/ml. *Relative expression of reIL-1β-stimulated COX-2 in chondrocytes treated with 10^{-5} and $10^{-6}M$ DEX was significantly ($P < 0.05$) less than that of cells stimulated with 5 ng of reIL-1β/ml.

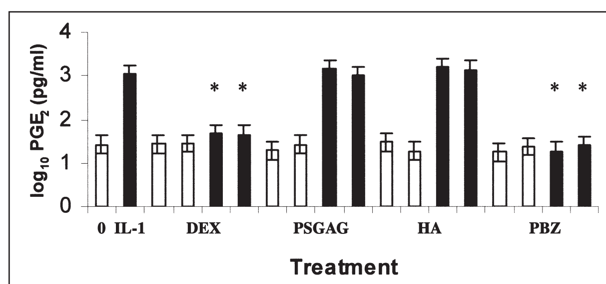

Figure 5—Concentration of prostaglandin E_2 (PGE_2) in conditioned media of equine chondrocytes obtained from grossly normal articular cartilage after incubation with various anti-arthritis preparations with or without 5 ng of reIL-1β/ml. Values are mean ± SEM $\log_{10}$ transformed PGE_2 concentrations from 3 experiments. *Concentration of PGE_2 in spent media from chondrocytes treated with both concentrations of DEX plus reIL-1β and both concentrations of PBZ plus reIL-1β are significantly ($P < 0.05$) less than that of cells stimulated with 5 ng of reIL-1β/ml. *See* Figure 4 for key.

determine relative abundance of mRNA. Standardization against corresponding values for ethidium bromide-stained 28S rRNA enabled semiquantitative evaluation of specific mRNA (ie, expression of COX-2 was calculated as the ratio of the intensity of the COX-2 bands to the intensity of the 28S rRNA band).

Analysis of data—Means for relative expression of COX-2 as well as $\log_{10}$-transformed concentrations of PGE_2 in media were compared by use of a 2-way ANOVA (blocked on the basis of horse) followed by a Bonferroni test to compare means for each treatment to that of the positive control sample. A value of $P < 0.05$ was considered significant.

Results

Amplification of cDNA—The PCR-amplified equine product corresponded to the expected 776-bp length. Single-pass sequencing revealed that there were no nucleotide differences for 450 bp with that of a published equine COX-2 sequence.[16] This confirmed that the cloned fragment was a portion of equine COX-2.

Stimulation of chondrocytes with reIL-1β—Northern blot analysis accomplished by use of an equine COX-2 probe hybridized to mRNA from chondrocytes exposed to reIL-1β for various periods revealed marked induction of a 4.5-kb COX-2 transcript after incubation for 6 hours (**Fig 1**). Resting expression of COX-2 was weak; however, graduated doses of reIL-1β induced noticeable dose-dependent induction of the gene (**Fig 2**). The dose of 5 ng/ml produced a submaximal response, and this dose was used for subsequent experiments.

Effects of anti-inflammatory compounds on COX-2 expression—None of the anti-arthritis compounds significantly influenced resting expression of COX-2. Use of DEX at both concentrations (ie, 10^{-6} and $10^{-5}M$) significantly reduced reIL-1β-induced upregulation of COX-2 expression (**Fig 3 and 4**). However, none of the other anti-arthritis preparations

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significantly affected COX-2 expression in cytokine-stimulated cultures.

Concentration of PGE_2 in conditioned medium— Concentrations of PGE_2 were determined by use of conditioned medium of cultures of chondrocytes obtained from 3 horses. These values were not normally distributed; thus, the statistical analysis was conducted on log_{10}-transformed values. Recombinant equine IL-1β-treated chondrocytes released significantly greater amounts of PGE_2 to the media than did control cultures. This increase in PGE_2 release was reduced by incubation with DEX and PBZ; however, incubation with PSGAG or HA did not appear to exert a significant effect on PGE_2 concentrations in cytokine-stimulated cultures (**Fig 5**).

Discussion

Prostaglandin E_2 is the most abundant of the prostanoids synthesized by articular tissues and has been implicated in a number of elements in the pathogenesis of osteoarthritis.[5-9] Given the importance of COX in the regulation of the arachidonic acid cascade, we believed that characterization of the expression and activity of the inducible form of this enzyme in equine chondrocytes and the potential modifying influences of commonly used anti-inflammatory preparations was warranted.

We prepared a cDNA probe by use of primers that were based on the published sequence of the human COX-2 gene. The nucleotide sequence of the obtained fragment matched that of an equine cDNA,[16] indicating that we successfully cloned a portion of the equine chondrocytic COX-2. Paralleling the findings of Boerboom and Sirois,[16] our probe hybridized with a COX-2 transcript of approximately 4.5 kb, the size of which exceeds that of the reported cDNA (3.4 kb). This disparity between the reported cDNA sequence (GenBank accession No. AF027334) and the size suggested by electrophoretic results has been attributed to the possibility of the former having been reverse transcribed from an internal poly(A)+ sequence in the 3'-untranslated region instead of the poly(A)+ tail.[16]

Chondrocytic expression of COX-2 was studied by use of equine chondrocytes propagated in high-density short-term confluent monolayer cultures subjected to a brief incubation in serum-free medium. Although chondrocytes in stationary monolayers do not closely mimic the chondrocytic environment in normal cartilage in vivo, the culture system offers the advantages of a uniform distribution of chondrocytic phenotype, facilitates quantification of biosynthetic activities by cell numbers, and permits the subjective evaluation of cytotoxic events by visual inspection. A brief period of culture in serum-free conditions was used to reduce the stimulatory effect of serum components on COX expression and to reduce confounding influences that serum may have had on responses to IL-1 or any of the anti-arthritis medications.

Similar to other reports[17-21] of experiments that used other tissues, we documented noticeable induction of COX-2 expression and secretion of PGE_2 in equine chondrocytes stimulated with nanogram quantities of IL-1β. Similar to observations for human chondrocyte cultures,[22] induction of equine COX-2 by reIL-1β was rapid and relatively long-lived. Specifically, high amounts of COX-2 transcript were detectable after 6 hours of incubation, and concentrations exceeding that of nonstimulated control cultures persisted for 72 hours in chondrocytes stimulated with 10 ng of reIL-1β/ml. Persistent induction of equine COX-2 mRNA contrasts with the findings of Morisset et al,[17] who observed transient induction of COX-2 in bovine chondrocytes with a return to resting concentrations at 24 hours of incubation. Although this finding may merely reflect differences in experimental conditions, it may also underscore the importance of species-specific investigations of expression and activity of central mediators of the osteoarthritic process.

Unique among the anti-inflammatory drugs tested, DEX inhibited gene expression of chondrocyte COX-2. Glucocorticoid abrogation of IL-1-induced stimulation of COX-2 expression appears to be a consistent phenomenon involving diverse cell types in a number of species.[1,17,19-21,23] It is likely that DEX inhibits equine chondrocytic COX-2 expression by several mechanisms that reduce transcription and mRNA stability, the initial details of which have been elucidated for other cell types.[24-27] Reduced gene expression of COX-2 is not the unique mechanism involved in glucocorticoid-induced inhibition of PGE_2 synthesis. For example, it has been reported[28-30] that DEX has transcriptional and posttranscriptional effects on expression of phospholipase A2 that may vary depending on the specific inflammatory stimulus and cell type. Mechanisms for regulation of members of the arachidonic acid cascade appear to vary between species and among cell types.[31]

Despite a lack of knowledge concerning its specific mode of action, HA is commonly used in the treatment of humans and horses with osteoarthritis. The reduced pain and improved articular mobility attributed to treatment with HA result from purported anti-inflammatory effects that may be physical or pharmacologic in nature.[32-35] In contrast to our data in which we did not detect an effect on COX-2 expression or PGE_2 synthesis, HA can inhibit PGE_2 synthesis in certain cell lines.[36-38] The means by which HA suppresses PGE_2 synthesis are unclear; however, there is evidence for a concentration- and molecular weight-dependent inhibition of the release of arachidonic acid from membrane phospholipids of human synovial fibroblasts.[39] Paralleling observations for corticosteroids, it is likely that additional studies will identify important differences in the effects of HA on various cell types. Analysis of our data suggests a limited effect of HA on PGE_2 synthesis by equine chondrocytes; differing results may be realized for similar experiments conducted on synovial fibroblasts.

Similar to the results for HA, PSGAG did not influence synthesis and release of PGE_2 by reIL-1β-stimulated chondrocytes and did not have an influence on COX-2 gene expression. Antiprostaglandin activity has been advanced as a means by which this compound contributes to palliative relief in animals with osteoarthritis, and inhibition of prostanoids is a docu-

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mented in vitro effect for PSGAG.[40] Although PSGAG is capable of pretranslational regulation of protein synthesis in that it reduces expression of inducible nitric oxide synthase in similar experimental conditions,[41] a significant effect on COX-2 mRNA concentrations was not detected in the study reported here. It is possible that if different conditions had been used, PSGAG may have inhibited COX or other members of the arachidonic acid cascade in chondrocytes or other articular cells.

Phenylbutazone is a competitive antagonist of COX.[42] As expected, PBZ drastically reduced the accumulation of PGE_2 in conditioned medium of reIL-1β-stimulated chondrocytes. Given this widely accepted mechanism of action of this NSAID, it was not surprising that transcriptional regulation of the COX-2 gene was not detected. Nonetheless, the hypothesis that NSAID can influence gene expression is not without precedent. For example, the NSAID indomethacin is capable of reducing concentrations of resting mRNA of matrix metalloproteinase-3 (stromelysin) as well as inhibiting reductions in cartilage proteoglycan synthesis that follow exposure of cultures of human articular chondrocytes to IL-1, suggesting that at least some NSAID are capable of regulating the expression of genes coding for proteins induced in the chondrocytes of diseased joints.[43,44]

[a]pCR 2.1-TOPO, Invitrogen, Carlsbad, Calif.
[b]Prostaglandin E_2 immunoassay, R&D Systems, Minneapolis, Minn.
[c]SigmaPlot, Jandel Corp, Chicago, Ill.
[d]DIG high prime DNA labeling and detection starter kit II, Roche Molecular Biochemicals, Indianapolis, Ind.
[e]Scion Image Beta 4.02, Scion Corp, Frederick, Md.

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