Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 142-148

NITRIC OXIDE MEDIATES SUPPRESSION OF CARTILAGE PROTEOGLYCAN SYNTHESIS BY INTERLEUKIN-1

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Received February 14, 1994

Slices of rabbit articular cartilage synthesized large quantities of nitric oxide (NO) following exposure to human recombinant interleukin-1β (hrIL-1β) or rabbit synovial cytokines (CAF). Each of these stimuli also strongly suppressed the biosynthetic incorporation of $^{35}SO_4^{2-}$ into the glycosaminoglycans (GAGs) of cartilage proteoglycans. Treatment of cartilage fragments with L-N^G-monomethylarginine (L-NMA), a competitive inhibitor of NO synthase, both inhibited NO synthesis in response to IL-1 and CAF and restored proteoglycan synthesis. D-NMA was inactive in this regard, and L-arginine reversed the effects of L-NMA. S-nitrosylacetylpenicillamine (SNAP), an organic donor of NO, reversibly mimicked the effect of IL-1 and CAF on $^{35}SO_4^{2-}$ incorporation. These data suggest that endogenously synthesized NO is the mediator which reduces cartilage proteoglycan synthesis in response to cytokines such as IL-1 and CAF. Antagonists of NO production may promote cartilage matrix synthesis and thus have potential as chondroprotective or chondroreparative agents.

Loss of articular cartilage is the major pathological lesion common to all arthritides. Although often asymptomatic until late stage disease, it results in joint failure; the surgical insertion of a prosthetic joint replacement is presently the only clinical recourse in such cases. Protection of the cartilage (chondroprotection) is thus an attractive pharmacologic goal.

The amount of articular cartilage at any given time is a function of the rate at which the cartilaginous matrix is degraded and the rate at which it is synthesized. Depletion of cartilage can occur through increased breakdown of the matrix, decreased

ABBREVIATIONS: nitric oxide - NO; human recombinant interleukin-1β - hrIL-1β; chondrocyte activating factors - CAF; glycosaminoglycan - GAG; N^G-monomethylarginine - NMA; S-nitrosylacetylpenicillamine - SNAP; inducible nitric oxide synthase - iNOS.

0006-291X/94 $5.00

synthesis or both. *In vitro* studies using fragments of bovine, porcine and lapine cartilage suggest that both mechanisms may be operative. Thus, interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), two cytokines present in arthritic joints, both increase the breakdown of the cartilaginous matrix while inhibiting its synthesis (1-4). Human cartilage seems to respond rather differently in that it mounts a poor catabolic response to IL-1 and TNF-α; instead, the major response is suppression of matrix synthesis (5,6). This suggests that in human joint disease, inhibition of matrix synthesis may be of major importance to the loss of articular cartilage. Thus the mechanisms through which this occurs merits particular attention.

As first shown by Stadler et al (7), articular chondrocytes produce large amounts of the free radical nitric oxide (NO) in response to IL-1 (7-10). Because of this, we have initiated a research program designed to investigate the role of NO in cartilage metabolism (7,10,11). For the reasons alluded to above, it seems of some importance to determine whether NO is involved in the suppressive effect on cartilage proteoglycan synthesis exerted by cytokines such as IL-1. Although this effect of IL-1 is well established, and may involve decreased expression of the aggrecan core protein gene (12), few mechanistic details are available.

In the present study we report data consistent with the hypothesis that NO is a major mediator of the suppressive effect of IL-1 upon proteoglycan synthesis by rabbit articular chondrocytes. In addition to using human, recombinant IL-1β (hrIL-1β) we have also tested the effects of a partially purified preparation of cytokines secreted by rabbit synovial fibroblasts. This material, known as "CAF" (chondrocyte activating factors; 13,14) is more physiological than purified hrIL-1β and, in the present experiments, does not cross species barriers.

METHODS

Cartilage Culture: New Zealand white rabbits (5-6 lbs) were killed, and the articular cartilages of the knee and shoulder joints removed by sterile dissection. Pooled cartilage was cut into fragments of approximately 3mm in length and 20mg per well placed into 24-well plates with 1ml of Neuman-Tytell medium (GIBCO, Long Island, NY) in the presence or absence of 20U/ml hrIL-1β (a generous gift of Elizabeth Arner, DuPont-Merck, Wilmington, DE), CAF (50μl), L-NMA, D-NMA (1mM), L-arginine (5mM) or SNAP (10μM-1mM), as indicated in the text. The L- and D-NMA used in this study was synthesized by Dr. Paul Dowd and Wei Zhang, Department of Chemistry, University of Pittsburgh. Dr. David Geller, Department of Surgery, University of Pittsburgh School of Medicine, kindly provided the SNAP.

Measurement of Proteoglycan Synthesis: Proteoglycan synthesis was measured as the incorporation of $^{35}SO_4^{2-}$ into glycosaminoglycans (GAG). To measure GAG synthesis 40μCi/well $Na_2{}^{35}SO_4$ (550 mCi mmole^{-1}; DuPont, Wilmington, DE) was added 24h after the introduction of cytokines, SNAP, NMA, or arginine. Following 24h further

incubation, the presence of ^{35}S labelled GAG was measured in the conditioned media and the cartilage fragments. For this purpose, conditioned media were passed through a P-10 column (Pharmacia, Piscataway, NJ) in 4M guanidinium hydrochloride, 50mM sodium sulfate, 50mM trizma buffer pH 7. The large proteoglycan molecules were excluded from this column and were collected in the void volume, whereas unincorporated $^{35}SO_4^{2-}$ eluted later. Proteoglycans were extracted from cartilage fragments by treatment with 0.5M NaOH for 48h and the ^{35}S present in the macromolecular fraction measured following separation by a P-10 column.

Other Methods: CAF is a partially purified mixture of rabbit synovial cytokines prepared from medium conditioned by activated cells of the HIG-82 rabbit synoviocyte line (15). Its preparation has been described elsewhere (14). CAF contains IL-1α, basic fibroblast growth factors, transforming growth factor-β and other, less well characterized cytokines (14).

Nitrite concentrations in conditioned media were determined by a spectrophotometric method based upon the Greiss reaction (16). We have previously shown that NO_2^- represents approximately 50% of the total nitrogen oxides generated from NO in cultures of rabbit articular chondrocytes, and that this percentage is stable over a wide range of NO concentrations (7).

Statistical Analysis: Each experiment used duplicate samples and each was repeated three times. Pooled data were used to generate the data presented here (i.e. n=6). Student's t-test for unpaired data was used for statistical analysis and $P \leq 0.05$ was considered significant.

RESULTS

Cartilage cultures spontaneously produced only low amounts of NO (Figs. 1-3). Addition of hrIL-1β (20U/ml) or CAF (50μl) provoked a large increase in NO synthesis which was accompanied by a 60-80% drop in the metabolic incorporation of $^{35}SO_4^{2-}$ into GAG (Figs. 1-3). Inhibition of GAG synthesis was noted with both the proteoglycans released into the culture medium and those retained within the cartilage fragments (data not shown). Addition of L-NMA strongly inhibited the production of NO and also substantially relieved the suppression of GAG synthesis (Fig. 1).

To provide a direct demonstration that NO inhibits proteoglycan synthesis by cartilage, we used SNAP which spontaneously releases NO in aqueous solution. As shown in fig. 2, 10μM SNAP released small amounts of NO, measured as nitrite, into the cultures and caused a modest, 22% reduction in ^{35}S incorporation. Because of biological variability, this reduction was not statistically significant. However, 100μM SNAP released high amounts of NO resulting in a much larger, statistically significant reduction in GAG synthesis of 60%, approaching that found with hrIL-1β (Fig. 2).

As fig. 2 shows, the very high levels of NO generated by 100μM SNAP failed to suppress GAG synthesis to a greater degree than hrIL-1β, where NO levels were lower. This suggests either that GAG synthesis is more susceptible to endogenously generated NO than to exogenously supplied NO, or that maximum suppression already achieved by levels of NO generated by 20U/ml hrIL-1β. Alternatively, these data may reflect kinetic

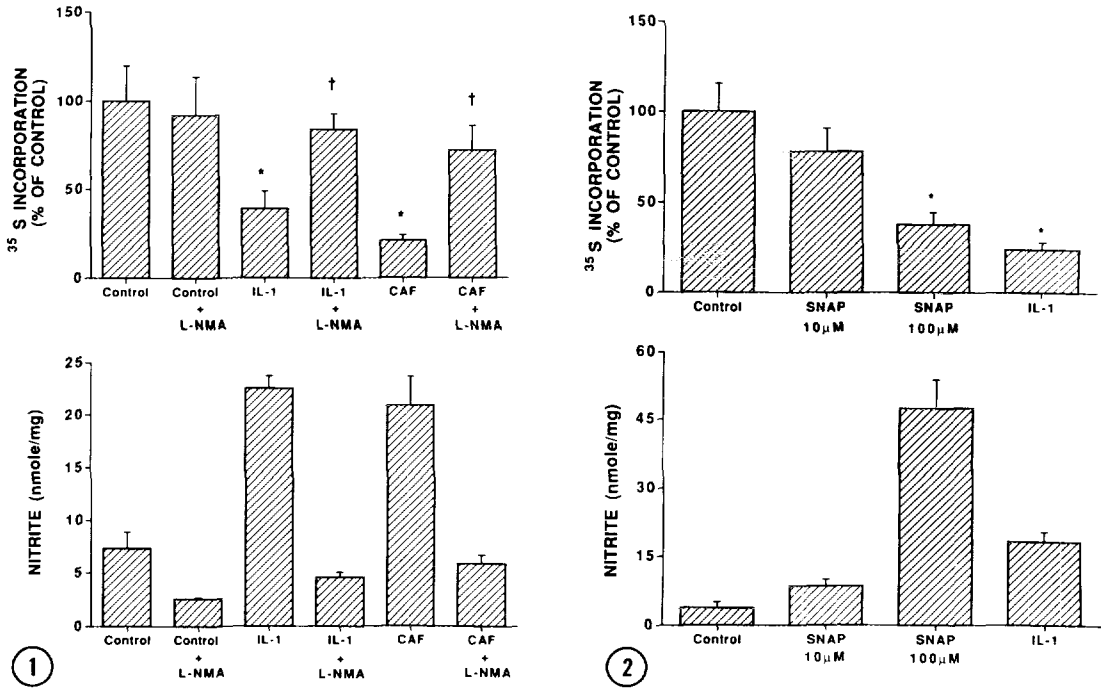

FIG. 1. **Effect of IL-1 and CAF on sulfate incorporation (top panel) and NO production (bottom panel) by rabbit articular cartilage.**

Values shown are means ±S.E. (n=6). * - statistically significant difference from control ($p<0.05$). † - statistically significant difference from corresponding culture lacking L-NMA ($p<0.05$).

FIG. 2. **Effect of SNAP on sulfate incorporation (top panel) and nitrite content of medium (bottom panel).**

Values shown are means ±S.E. (n=6). * - statistically significant difference from control ($p<0.05$).

differences between the generation of NO by SNAP, which has a half-life of 4-6 hours, and by chondrocytes which produce NO continuously.

The suppressive effects of both 10μM and 100μM SNAP were reversed by placing the cartilage fragments in fresh medium lacking SNAP (data not shown). This suggests that the inhibition of GAG synthesis is not due to toxicity. However, the suppressive effects of 1mM SNAP upon GAG synthesis were not reversed by placement in fresh medium (data not shown).

Two control experiments were performed to confirm the specificity of the L-NMA. Firstly, D-NMA, the biologically *in*active enantiomer, failed to inhibit NO synthesis or to alleviate the suppression of GAG synthesis (Fig. 3). Secondly, L-arginine, the substrate for NO synthase with which L-NMA competes when inhibiting the enzyme, was able to reverse the effects of L-NMA upon GAG synthesis (Fig. 3). L-arginine

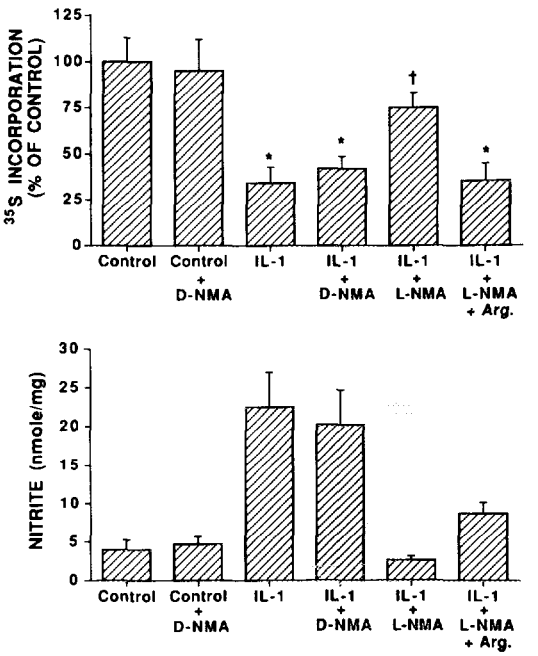

FIG. 3. **Effects of L-NMA, D-NMA and L-arginine on sulfate incorporation (top panel) and NO production (bottom panel) by rabbit articular cartilage.** Values shown are means ±S.E. (n=6). * - statistically significant difference from control ($p<0.05$). † - statistically significant difference from corresponding culture lacking L-NMA or L-arginine ($p<0.05$).

alone had only a small effect on GAG synthesis and NO production (data not shown). Thus the most likely explanation of the data shown in figure 1 is that L-NMA blocks the suppressive effect of hrIL-1β and CAF upon GAG synthesis by inhibiting NO synthase.

DISCUSSION

Increasing evidence implicates NO in the pathophysiology of arthritis (11). Although details are lacking, there are grounds for suggesting a role for NO in both the inflammation and chondrodepletion that occurs in this disease (11; 17-19). The present data support the hypothesis that NO mediates the suppression of cartilage matrix synthesis occurring in response to intraarticular cytokines. Inhibition of NO synthesis by chondrocytes may thus have a chondroprotective effect in human joint disease where, as described in the introduction, cartilage may be particularly vulnerable to the suppressive effects of cytokines on GAG synthesis. In rat adjuvant and streptococcal cell wall arthritis, administration of L-NMA indeed provided strong chondroprotection (17,19), although this may have been secondary to its antiinflammatory effect.

Substances which inhibit the production of NO by articular chondrocytes could serve as the basis for future chondroprotective drugs. Clearly, such an endeavor would be best served by agents with selectivity for chondrocytes. Chondrocytes possess the inducible form of NO synthase (iNOS; 20). Although it should prove possible to design inhibitors which preferentially block iNOS rather than the constitutive forms of the enzyme, there appears to be only one gene for iNOS (21,22). On this basis, it may be difficult to suppress specifically the synthesis of NO by chondrocytes, unless differential post-translational modification occurs. However, we have noted that the inhibition profile for NO synthesis by cultures of articular chondrocytes differs from that of other cells of the same species that also possess iNOS (our unpublished data). Idiosyncracies in the metabolism of chondrocytes may thus provide a selectivity lacking in the purified enzyme. As an alternative to the use of iNOS inhibitors, NO production could be down-regulated biologically by, for instance, the use of certain cytokines. IL-4, IL-10 and TGF-β have been shown to down-regulate iNOS in certain types of cells (23-25).

The observations reported in this communication open new approaches with which to address the problem of cartilage matrix depletion.

ACKNOWLEDGMENTS

Supported, in part, by grants from the Ferguson Foundation and the Western Pennsylvania Chapter of the Arthritis Foundation. We are grateful to Dr. Teresa Morales, NIH, for instruction in several of the methods used in this study. Dr. Paul Dowd and Wei Zhang, Department of Chemistry, University of Pittsburgh are thanked for synthesizing the L-NMA and D-NMA, Liz Arner for supplying the hrIL-1β and Dr. David Geller for providing the SNAP.

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