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Nitric oxide and proteoglycan biosynthesis by human articular chondrocytes in alginate culture

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Abstract Interleukin-1α and β induced the production of large amounts of nitric oxide by normal, human articular chondrocytes in alginate culture; at the same time the biosynthesis of proteoglycan was strongly suppressed. In a dose-dependent manner, N^G-monomethyl-L-arginine both inhibited nitric oxide formation and relieved the suppression of proteoglycan synthesis. However concentrations of N^G-monomethyl-L-arginine which completely prevented nitric oxide production only partially restored proteoglycan biosynthesis, even at low doses of interleukin-1 where suppression of proteoglycan synthesis was modest. The organic donor of nitric oxide, *S*-nitrosyl-acetyl-D,L- penicillamine also inhibited proteoglycan biosynthesis, but not as extensively as interleukin-1. These data suggest that interleukin-1 suppresses synthesis of the cartilaginous matrix through more than one mechanism, at least one of which is dependent upon the production of nitric oxide.

Key words: Nitric oxide; Interleukin-1; Chondrocyte; Proteoglycan synthesis; Arthritis; Human articular cartilage

1. Introduction

Interleukin-1 (IL-1) strongly suppresses the synthesis of proteoglycan (PG) molecules by normal, human, articular cartilage without eliciting a marked catabolic response ([1,2]; manuscript in preparation).

As IL-1 is synthesized within arthritic joints [3,4] it is likely to contribute to the loss of articular cartilage in arthritis by inhibiting synthesis of the cartilaginous matrix. The sensitivity of chondrocyte matrix synthesis to IL-1 compromises the inherent repair capacity of cartilage. The ability of articular chondrocytes to regenerate lost matrix may play a key role in preventing harmful pathological changes in the cartilage surface which lead to osteoarthritis. The mechanism through which IL-1 inhibits PG synthesis by articular chondrocytes is unknown.

IL-1 also up-regulates expression of the inducible form of nitric oxide synthase (iNOS) within articular chondrocytes [5], leading to the synthesis of large amounts of nitric oxide (NO) [6–9]. Indeed, synthesis of NO under these conditions is so great that it is unlikely to be irrelevant to the physiology of articular cartilage. However, almost nothing is known of the role that NO plays in chondrocyte metabolism.

In the present investigation, we have sought to determine the degree to which NO mediates the suppressive effect of IL-1 upon PG biosynthesis by normal human, articular chondrocytes. These studies were facilitated by the use of the alginate culture system, within which chondrocytes retain their differentiated phenotype [10] and show almost identical behaviour to cartilage slice cultures when treated with IL-1 ([1]; manuscript in preparation).

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Abbreviations: hrIL-1, human, recombinant interleukin-1; hrIL-1ra, human, recombinant interleukin-1 receptor antagonist; NMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PG, proteoglycan; SNAP, *S*-nitrosyl-acetyl-D,L-penicillamine.

2. Materials and methods

2.1. Reagents

Pronase was purchased from Calbiochem, La Jolla, CA; collagenase (*Clostridium histolyticum*, type CLS-2) from Cooper Biomedicals, Cappel Worthington, Malvern, PA; Fetal bovine serum (FBS) was from Hyclone, Logan, UT; Ham's F12/DMEM medium from Gibco, Grand Island, NY; and low viscosity alginate (Keltone LV) from Kelco, Chicago, IL. Sephadex G-25 (as prepacked PD-10 columns) was from Pharmacia, Piscataway, NJ. Radiolabelling was performed with [^{35}S]sulphate, 25–40 Ci/mg, purchased from Amersham, Arlington Heights, IL. N^G-monomethyl-L-arginine (L-NMA) and N^G-monomethyl-D-arginine (D-NMA) were kindly synthesized by Dr. Paul Dowd and Wei Zhang, Department of Chemistry, University of Pittsburgh, *S*-nitrosyl-acetyl-D,L-penicillamine (SNAP) purchased at Alexis Corp., Läufelingen, Switzerland, human recombinant interleukin-1α and β (hrIL-1α/β) (Dr. Vosbeck, Ciba-Geigy, Basle) were a gift from Dr. P. Lötscher, Department of Rheumatology University Hospital, University of Berne, Switzerland, human recombinant interleukin-1 receptor antagonist (hrIL-1ra) was purchased at R&D Systems Europe Ltd., Abington, UK. Bisbenzimidazole fluorescent dye (Hoechst dye #33258) was purchased from Polysciences, Warrington, PA, papain, L-arginine and calf thymus DNA were from Sigma, St. Louis, MO. All other chemicals were reagent grade and purchased from several different companies.

2.2. Cartilage sampling and isolation of chondrocytes

Fresh human articular cartilage was obtained post-mortem from three organ donors between 39 and 55 years of age with no history of joint problems. Macroscopically normal cartilage from the weight-bearing parts of the medial and lateral femoral condyles were dissected under sterile conditions and immediately placed in cold Ham's F-12/DME (Dulbecco's modified Eagle medium) medium supplemented with 5% fetal bovine serum (FBS).

Chondrocytes were isolated from the harvested slices of human articular cartilage by digestion of the extracellular matrix as published previously [11].

2.3. Cell culture in alginate beads

Chondrocytes were encapsulated and cultured in alginate beads as recently described by Guo et al. [12] and slightly modified by Häuselmann et al. [11]. Briefly, the isolated cells were suspended in sterile 0.15 M NaCl containing low viscosity alginate gel (1.2%) at a density of 4×10^6 cells/ml of gel, then slowly expressed through a 22-gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution. After instantane-

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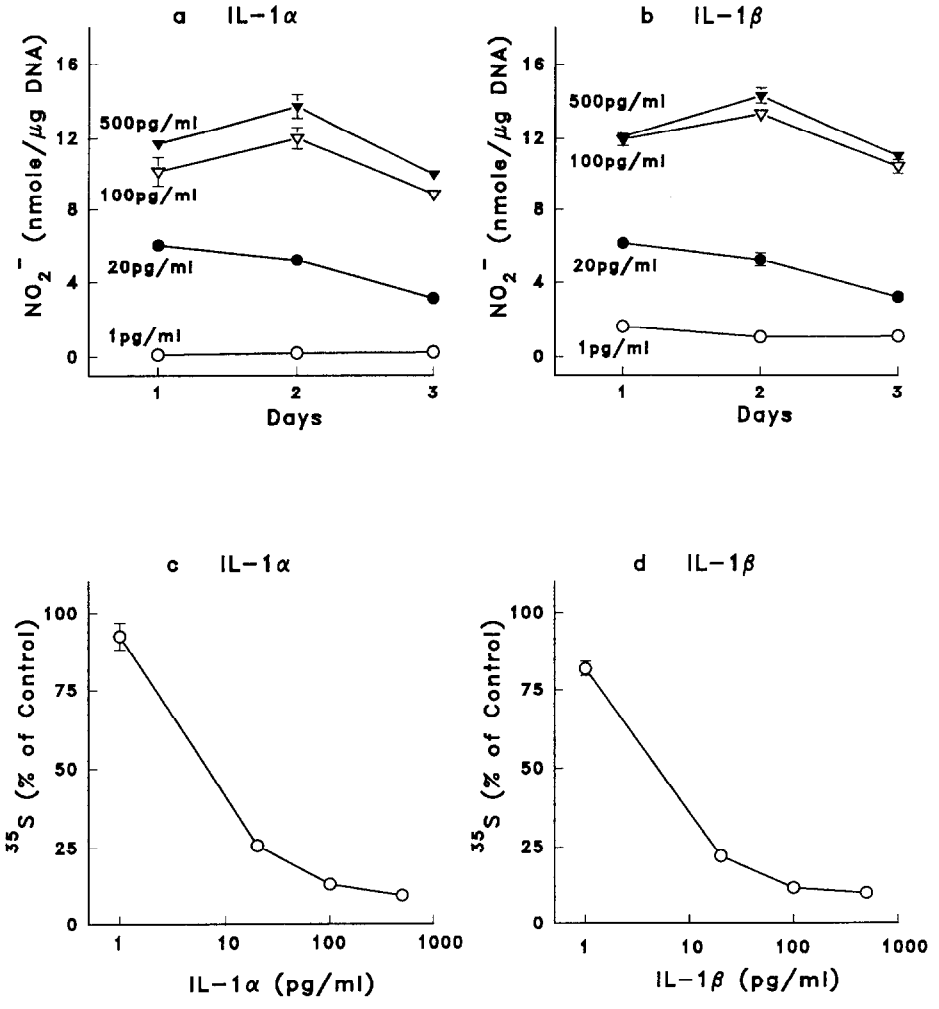

Fig. 1. Effect of hrIL-1α and β on the biosynthesis of NO and sulphated proteoglycan by human articular chondrocytes in alginate cultures. (a,b) NO_2^- content (nmol/μg DNA) of culture supernatants of human articular chondrocytes, after 1, 2 and 3 days of treatment with different doses of hrIL-1α and β ($n = 4$). (c,d) Inhibition of $^{35}SO_4^{-2}$ incorporation into proteoglycan macromolecules by human chondrocytes, after treatment for 3 days with different doses of hrIL-1α and -β, expressed as % of control ($n = 3$). x-Axes of Fig. 1c and 1d have logarithmic scales.

ous gelation, the beads were allowed to polymerise further for a period of 10 min in the $CaCl_2$ solution. After 1 wash in 10 volumes of 0.15 M NaCl and 3 washes in 10 volumes of Ham's F12/DME medium, the beads finally were placed in 24 well plates and maintained by feeding daily 0.6 ml/well of Ham's F-12/DME medium containing 50 μg/ml gentamicin, 25 μg/ml ascorbic acid and 10% FBS. The cultures were incubated at all stages in a humidified atmosphere of 5% CO_2 in air at 37°C.

2.4. Treatment of human chondrocytes in culture with hrIL-1α/β, hrIL-1ra, L-NMA, D-NMA, L-arginine and SNAP

Seven days after encapsulation, the cells were treated daily for 3 days with the following agents either singly or in various combinations: 1–500 pg/ml hrIL-1α/β; 500 ng/ml hrIL-1ra; 0.01–1mM L-NMA; 1 mM D-NMA; 1–100 mM L-arginine and 0.01 mM–1 mM SNAP. Other cultures were maintained as controls without additions. For each treatment regimen or control 4 culture wells with 5 beads/well were used.

2.5. Proteoglycan biosynthesis

After 3 days of treatment, $Na_2{}^{35}SO_4$ (20 μCi/ml final concentration) was added to the appropriate wells in triplicate and incubated for 4 h. After this period the labelling medium and alginate beads were extracted at 4°C for 48 h by addition of an equal volume of 8 M guanidine hydrochloride (GuHCl), 20 mM EDTA and a mixture of proteinase inhibitors [14]. For quantitative evaluation of ^{35}S-labeled PGs, aliquots of the stored extracts were eluted on Sephadex G-25M in PD 10 columns under dissociative conditions [14]. The radioactivities of the newly synthesized PGs were measured by scintillation counting.

2.6. Determination of DNA content in chondrocyte cultures

DNA contents of the cultured chondrocytes were quantified using the bisbenzimidazole fluorescent dye, Hoechst 33258 [13]. Alginate cultures (one well per group), treated as mentioned above, were digested with papain (125 μg/ml) and evaluated in a spectrofluorometer as published earlier by Aydelotte and Kuettner [14].

2.7. Measurement of nitrite

We have previously determined that NO generated by cultures of articular chondrocytes forms equimolar amounts of NO_2^- and NO_3^- as its stable in vitro end products [6]. NO production was thus measured as NO_2^- formation using a spectrophotometric assay based upon the Griess reaction [15]. Nitrite concentration was measured in conditioned medium of quadruplicate cultures per group after 24, 48 and 72 h of treatment .

3. Results and discussion

Addition of hrIL-1α or hr IL-1β to human articular chondrocytes in alginate culture induced a dose-dependent production

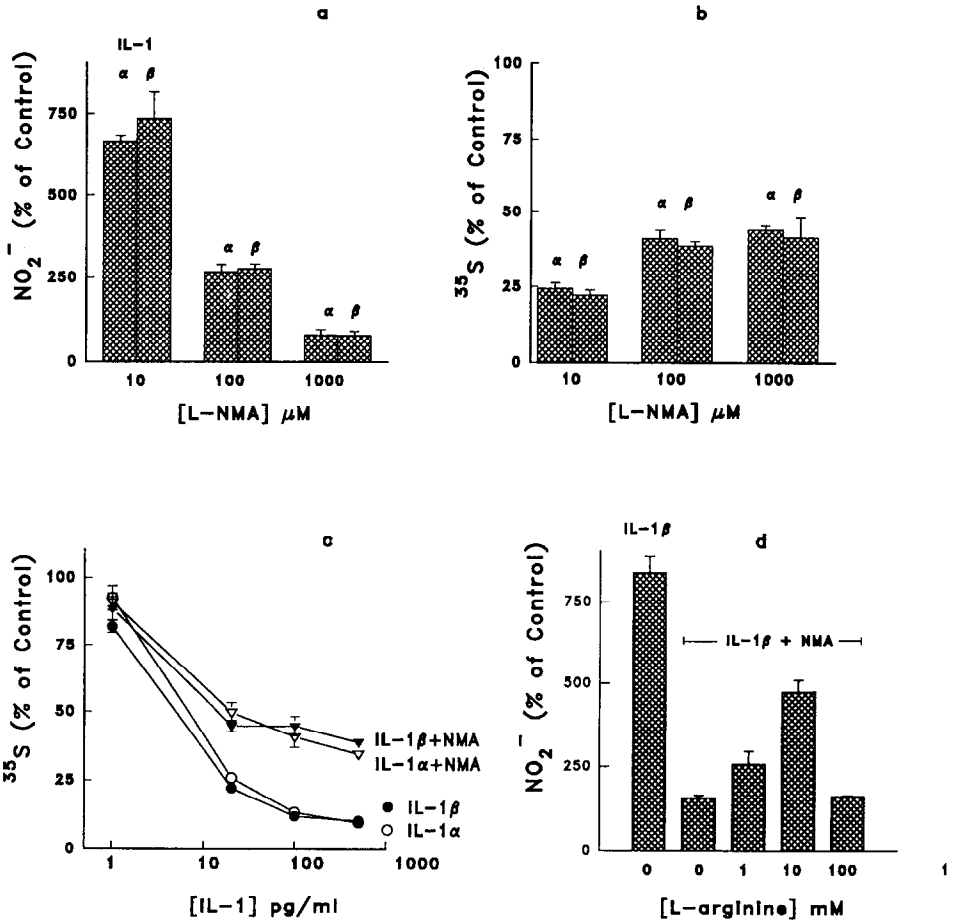

Fig. 2. Effect of L-NMA on the biosynthesis of NO and sulphated proteoglycans by human articular chondrocytes in alginate culture in the presence or absence of hrIL-1α or -β (a) NO_2^- released into the culture supernatants (nmol/μg DNA) by human chondrocytes after treatment for 2 days with different doses of L-NMA in the presence of 500 pg/ml hrIL-1α or -β, expressed as % of control. (b) $^{35}SO_4^{-2}$ incorporation into proteoglycan macromolecules (cpm/μg DNA) of human chondrocytes after treatment for 3 days with different doses of L-NMA in the presence of 500 pg/ml hrIL-1α or -β, expressed as % of control. (c) Inhibition of $^{35}SO_4^{-2}$ incorporation into proteoglycan macromolecules of human chondrocytes after treatment for 3 days with different doses of hrIL-1α or β in the presence or absence of 1 mM L-NMA. (d) NO_2^- released into the culture supernatants (nmol/μg DNA) by human chondrocytes after treatment for 2 days with different doses of L-arginine in the presence of 500 pg/ml hrIL-1β and 0.1 mM L-NMA expressed as % of control (first bar shows NO_2^- released in the presence of hrIL-1β alone). x-Axis in Fig. 2c has a logarithmic scale.

of NO which continued during each of the three days in culture and showed a maximal response after 48 h of treatment with higher doses of IL-1α/β (Fig. 1a,b). There was a corresponding dose-dependent inhibition of the incorporation of $^{35}SO_4^{-2}$ into PG (Fig. 1c,d). hrIL-1ra inhibited both NO production and the reduction in PG synthesis that occurred in response to hrIL-1α and hrIL-1β (Table 1). Interestingly, hrIL-1ra was significantly less effective ($P \leq 0.002$) in relieving the suppressive effect of IL-1β on PG synthesis than in relieving that of IL-1α, an effect which was paralleled by the highly significant differential behaviour of hrIL-1ra on the inhibition of NO production induced by the two IL-1 isoforms ($P \leq 0.0002$) (Table 1). The reason for this is unclear at present. However, it confirms other data of ours in experiments using the two isoforms of IL-1 on human articular cartilage slice cultures (manuscript in preparation).

Two lines of evidence suggest that NO production is mechanistically linked to the suppression of PG biosynthesis occurring in these cultures. Firstly, L-NMA, a competitive inhibitor of all isoforms of NOS which completely inhibited NO

Table 1
Effect of IL-1ra on the biosynthesis of nitric oxide and sulphated proteoglycans by human articular chondrocytes treated with hrIL-1α OR-β

Treatment	NO_2^- production (nmol/μg DNA)	PG-synthesis (^{35}S-cpm/μg DNA)
None	1.17 ± 0.24	21599 ± 1698
IL-1α	17.18 ± 0.14	2770 ± 58
IL-1β	17.32 ± 0.6	2439 ± 41
IL-1ra	1.91 ± 0.18	22923 ± 472
IL-1α + IL-1ra	1.6 ± 0.25] *	21469 ± 638] **
IL-1β + IL-1ra	3.73 ± 0.4]	16405 ± 785]

IL-1α and IL-1β were added at a final concentration of 0.5 ng/ml; IL-1ra was added at a final concentration of 500 ng/ml. Values represent NO_2^- production during the 24–48 h culture period. PG-synthesis denotes newly incorporated $^{35}SO_4^{-2}$ into proteoglycan macromolecules (cpm/μg DNA). Student's t-test for independent sets of data (cultures treated with IL-1α + IL-1ra in comparison with IL-1β + IL-1ra) was used for statistical analysis and $P \leq 0.05$ was considered significant.

*$P \leq 0.0002$; **$P \leq 0.002$; means ± S.D. ($n = 4$ for nitrite production; $n = 3$ for PG synthesis).

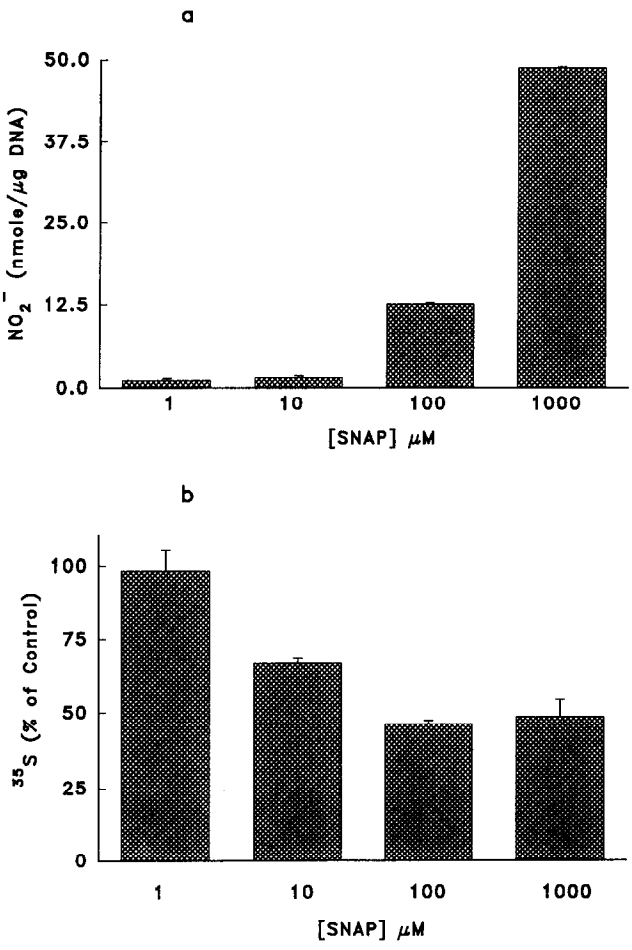

Fig. 3. Effect of SNAP on the biosynthesis of NO and sulphated proteoglycans by human articular chondrocytes in alginate cultures. (a) NO_2^- content of culture supernatants 2 days after addition of different doses of SNAP. (b) Inhibition of $^{35}SO_4^{-2}$ incorporation into proteoglycan macromolecules (cpm/μg DNA) after 3 days of treatment with different doses of SNAP, expressed as % of control.

production (Fig. 2a), partially restored PG biosynthesis (Fig. 2b) in a dose-dependent fashion. However, restoration of PG synthesis was never complete, even at low doses of IL-1 which inhibited PG synthesis only modestly (Fig. 2c). D-NMA failed to inhibit NO production and had no effect on PG biosynthesis either in the presence or absence of IL-1 (data not shown). Attempts were made to reverse the inhibitory effects of L-NMA by adding L-arginine. As shown in Fig. 2d, 1 mM and 10 mM arginine partially reversed the inhibitory effect of L-NMA on NO biosynthesis. But 100 mM arginine was inactive and apparently toxic. Similar toxicity has been noted previously in cultures of rabbit articular chondrocytes [6]. Such toxicity may help explain why arginine was unable to reverse the inhibition of PG synthesis in the present study (data not shown). This problem is exacerbated by the the high concentration of L-arginine in commercial culture medium (0–5 mM in DMEM; 1 mM in Ham's F12). These concentrations are far in excess of the normal serum level of arginine (≈100 μM).

The hypothesis that NO inhibits PG synthesis was supported by the ability of SNAP, a chemical donor of NO (Fig. 3a), to suppress PG biosynthesis. SNAP mimicked IL-1 in reducing PG biosynthesis by chondrocytes in a dose-dependent manner (Fig. 3b). The maximum inhibition produced by SNAP failed to achieve that produced by IL-1, even though the total amount of NO generated was four-fold higher. However, the inhibition of PG synthesis achieved by SNAP was approximately equal to the inhibition achieved by that component of the IL-1 effect which was sensitive to L-NMA (compare Figs. 2b and 3b).

Collectively, these findings suggest that IL-1 inhibits PG synthesis through more than one mechanism, not all of which require NO.

Similar data to ours have recently been communicated in abstract form by Hickery et al. [16], who studied slices of normal, human articular cartilage. Qualitatively similar findings have also been reported for lapine articular cartilage [17], suggesting that this might be a general mechanism of modulating cartilage matrix biosynthesis.

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