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Modulation of IL-1-induced cartilage injury by NO synthase inhibitors: a comparative study with rat chondrocytes and cartilage entities

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1 Nitric oxide (NO) is produced in diseased joints and may be a key mediator of IL-1 effects on cartilage. Therefore, we compared the potency of new [aminoguanidine (AG), S-methylisothiourea (SMT), S-aminoethylisothiourea (AETU)] and classical [N^{ω}-monomethyl-L-arginine (L-NMMA), N^{ω}-nitro-L-arginine methyl ester (L-NAME)] NO synthase (NOS) inhibitors on the inhibitory effect of recombinant human interleukin-1β (rhIL-1β) on rat cartilage anabolism. Three different culture systems were used: (1) isolated chondrocytes encapsulated in alginate beads; (2) patellae and (3) femoral head caps.

2 Chondrocyte beads and cartilage entities were incubated *in vitro* for 48 h in the presence of rhIL-1β with a daily change of incubation medium to obtain optimal responses on proteoglycan synthesis and NO production. Proteoglycan synthesis was assessed by incorporation of radiolabelled sodium sulphate [$Na_2{}^{35}SO_4$] and NO production by cumulated nitrite release during the period of study.

3 Chondrocytes and patellae, as well as femoral head caps, responded concentration-dependently to IL-1β challenge (0 to 250 U ml^{-1} and 0 to 15 U ml^{-1} respectively) by a large increase in nitrite level and a marked suppression of proteoglycan synthesis. Above these concentrations of IL-1β (2500 U ml^{-1} and 30 U ml^{-1} respectively), proteoglycan synthesis plateaued whereas nitrite release still increased thus suggesting different concentration-response curves.

4 When studying the effect of NOS inhibitors (1 to 1000 μM) on NO production by cartilage cells stimulated with IL-1β (25 U ml^{-1} or 5 U ml^{-1}), we observed that: (i) their ability to reduce nitrite level decreased from chondrocytes to cartilage samples, except for L-NMMA and AETU; (ii) they could be roughly classified in the following rank order of potency: AETU > L-NMMA $\geqslant$ SMT > AG $\geqslant$ L-NAME and (iii) AETU was cytotoxic when used in the millimolar range.

5 When studying the effect of NOS inhibitors on proteoglycan synthesis by cartilage cells treated with IL-1β, we observed that: (i) they had more marked effects on proteoglycan synthesis in chondrocytes than in cartilage samples; (ii) they could be roughly classified in the following rank order of potency: L-NAME $\geqslant$ L-NMMA > > AG > SMT > > AETU and (iii) potentiation of the IL-1 effect by AETU was consistent with cytotoxicity in the millimolar range.

6 D-isomers of L-arginine analog inhibitors (1000 μM) were unable to correct nitrite levels or proteoglycan synthesis in IL-1β treated cells. L-arginine (5000 μM) tended to reverse the correcting effect of L-NMMA (1000 μM) on proteoglycan synthesis, thus suggesting a NO-related chondroprotective effect. However, data with L-NAME and SMT argued against a general inverse relationship between nitrite level and proteoglycan synthesis.

7 Dexamethasone (0.1 to 100 μM) (i) failed to inhibit NO production in femoral head caps and chondrocytes beads whilst reducing it in patellae (50%) and (ii) did not affect or worsened the inhibitory effect of IL-1β on proteoglycan synthesis. Such results suggested a corticosteroid-resistance of rat chondrocyte iNOS. Data from patellae supported a possible contribution of subchondral bone in NO production.

8 In conclusion, our results suggest that (i) NO may account only partially for the suppressive effects of IL-1β on proteoglycan synthesis, particularly in cartilage samples; (ii) the chondroprotective potency of NOS inhibitors can not be extrapolated from their effects on NO production by joint-derived cells and (iii) L-arginine analog inhibitors are more promising than S-substituted isothioureas for putative therapeutical uses.

Keywords: Cartilage organ system; chondrocytes; proteoglycan synthesis; interleukin-1; nitric oxide; N^{ω}-monomethyl-L-arginine; N^{ω}-nitro-L-arginine methyl ester; aminoguanidine; S-methylisothiourea; S-aminoethylisothiourea

Introduction

Destruction of articular cartilage is a common end point of rheumatoid arthritis or osteoarthritis leading to joint disability and cartilage damage. Amongst inflammatory agents, cytokines, such as interleukin-1β (IL-1β) or tumor necrosis factor α (TNFα) (Van de Loo *et al.*, 1995), are able to impair cartilage homeostasis by disrupting the balance between anabolic and catabolic processes. Indeed, such cytokines can induce a suppression of cartilage anabolism by reducing the synthesis of the main extracellular matrix components such as collagens and proteoglycans. These cytokines also promote cartilage

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catabolism by inducing either the synthesis or the activation of matrix metalloproteinases (MMPs) or by reducing the level of tissue inhibitors of metalloproteinases (Arend & Dayer, 1990). Most of the drugs marketed to date, as nonsteroidal anti-inflammatory drugs and corticosteroids, are unable to prevent cartilage damage (Dingle, 1991). Therapeutic insights are obvious concerning cartilage catabolism since pharmacological targets, such as MMPs, have emerged the more so that MMPs inhibitors have shown a sparing effect in experimental models of osteoarthritis (O'Byrne *et al*., 1995). However, no major target has been pointed out for the anabolic process of cartilage. In this last decade, Nitrix Oxide (NO) has been increasingly proposed as a possible candidate since it is implicated in many pathophysiological processes and particularly in IL-1-mediated responses (Farrell & Blake, 1996).

NO, a free, highly reactive, radical, is generated from L-arginine by a family of enzymes; the Nitric Oxide Synthases (NOS). At least three distinct forms of NOS have been identified and characterized (Knowles & Moncada, 1994; Förstermann & Kleinert, 1995). The constitutive NOS (NOS_I or nNOS and NOS_{III} or eNOS) produce nanomolar amounts of NO which plays an important role in intracellular and intercellular signalling and is a key regulator of physiological processes. In contrast, inducible NOS (NOS_{II} or iNOS) can be induced in many cell types in response to pro-inflammatory cytokines and/or immunologic stimuli and generates micromolar quantities of NO for a long time period. The excess of NO produced by iNOS has been implicated in pathophysiological processes including septic shock and inflammatory diseases (Laskin *et al*., 1994; Farrell & Blake, 1996).

With special reference to joint disorders, several lines of evidence suggest that NO overload may be a common finding. Firstly, increased levels of nitrite have been observed in serum and synovial fluids of patients with either rheumatoid arthritis or osteoarthritis (Farrell *et al*., 1992; Stichtenoth *et al*., 1994; Ueki *et al*., 1996). Secondly, inflammatory cytokines, such as IL-1β or TNFα, have been found to promote the expression of iNOS in human joint-derived cells such as chondrocytes, synoviocytes and osteoblasts (Stadler *et al*., 1991; Palmer *et al*., 1993; Rediske *et al*., 1994; Grabowski *et al*., 1996). Thirdly, an increase in urinary nitrate excretion (Stefanovic-Racic *et al*., 1994, 1995; Cannon *et al*., 1996) and synovial iNOS mRNA expression (Cannon *et al*., 1996) were reported during the course of experimental polyarthritis. In addition, NOS inhibitors have shown anti-inflammatory effects in several experimental models suggesting that NO modulation may be of therapeutical relevance (Ialenti *et al*., 1992; McCartney-Francis *et al*., 1993; Stefanovic-Racic *et al*., 1994; 1995). Interestingly, the reduction of joint swelling was sometimes accompanied by a reduction of the severity of histological lesions within joint tissues (McCartney-Francis *et al*., 1993; Stefanovic-Racic *et al*., 1994). However, it is not possible to ascertain whether this beneficial effect comes from an anti-inflammatory effect rather than from a chondroprotective one.

Recent studies support the theory that NO may be a key mediator of IL-1 effects on cartilage. For example, studies of cartilage catabolism have shown that NO modulation, in response to IL-1 challenge, may affect metalloproteinase activities in bovine (Stefanovic-Racic *et al*, 1996) and human (Murrell *et al*., 1995) samples. However, this contribution of NO remains controversial since it can be considered as harmful (Murrell *et al*., 1995) or protective (Stefanovic-Racic *et al*., 1996; Haüselmann *et al*., 1998) for cartilage with variability being partly attributable to species differences. Concerning the inhibitory effect of IL-1 on cartilage anabolism, *in vitro* experiments using rabbit articular cartilage slices (Taskiran *et al*., 1994), rat femoral heads (Järvinen *et al*., 1995) or human chondrocytes encapsulated in alginate beads (Haüselmann *et al*, 1994) have suggested a possible involvement of NO. Such general meaning was supported by the apparent inverse relationship between NO production and proteoglycan synthesis and the ability of few NOS inhibitors (L-NMMA or L-NIO) to correct IL-1 effects. However, data were obtained in different experimental conditions, and they were not confirmed with bovine cartilage slices (Stefanovic-Racic *et al*., 1996). In addition, data on cartilage anabolism are lacking for NOS inhibitors with increased selectivity for the inducible isoform.

In the present study, we compared new and classical NOS inhibitors for their potency to improve the inhibitory effect of IL-1 on proteoglycan synthesis. Cartilage anabolism was studied in chondrocytes embedded in a simplified (alginate beads) or complex (patellae and femoral head caps) tridimensional matrix. The effect of recombinant human IL-1β (rhIL-1β) was optimized in each system in order to allow an accurate comparison of NOS inhibitors as regard to proteoglycan synthesis. Our results led to the conclusions that (i) the inhibitory effect of NOS inhibitors on nitrite levels generally decreased from chondrocytes to cartilage samples, possibly due to differences in drug diffusion, (ii) whatever the system used, inhibition of NO production did not account for the ability of NOS inhibitors to slow down the inhibitory effect of IL-1 on proteoglycan synthesis; (iii) L-NMMA and L-NAME were able to preserve proteoglycan synthesis after IL-1 challenge whereas inhibitors with selectivity for macrophagic inducible NOS were poorly effective or even toxic and (iv) the induction of NO production by IL-1 was somewhat resistant to corticosteroids in rat chondrocytes.

Methods

Chondrocyte isolation and culture in alginate beads

Normal articular cartilage was obtained from Wistar male rats (130–150 g) (Charles River, France) killed under dissociative anaesthesia [ketamine (Rhône-Mérieux, France) and acepromazine (Sanofi Santé Animale, France)]. After joint surgery, articular cartilage pieces were aseptically dissected from femoral head caps and chondrocytes were obtained by sequential digestion with pronase and collagenase (Boehringer Mannheim, Meylan, France) as previously described by Kuettner *et al*. (1982). Chondrocytes were further encapsulated in alginate beads according to the method of Haüselmann *et al*. (1992). Briefly, cells were suspended in sterile filtered low viscosity alginate solution (1.2%) at a concentration of 6×10^6 cells ml^{-1} and slowly expressed through a 22 gauge needle into a 102 mM $CaCl_2$ solution. After instantaneous gelation, the beads were allowed to further their polymerization for a 10 min period in $CaCl_2$ solution. After two washes in 0.15 M NaCl and one wash in DMEM/Ham's F12 medium supplemented with L-glutamine (2 mM), gentamycin (50 μg ml^{-1}), amphotericin B (0.25 μg ml^{-1}), heat inactivated foetal calf serum (FCS) (10%) (Dutscher, France) and ascorbic acid (25 μg ml^{-1}), beads were finally maintained in complete culture medium for 6 days in a humidified atmosphere of 5% CO_2 at 37°C before further experiments.

Cartilage sample collection and organ culture

As described above, patellae and femoral head caps were collected under sterile conditions and carefully dissected free of

residual surrounding tissue. Each sample was placed into a well of sterile 24-well plates containing 1 ml of phosphate buffered saline supplemented with penicillin (100 UI ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$) (PBS-Pen-Strep). Cartilage anatomical entities were washed once in PBS-Pen-Strep to remove any adhered blood clots. Thereafter, each patella and femoral head cap was placed into one well of sterile 24-well plates containing 1 ml RPMI Hepes 1640 medium supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), gentamycin (40 $\mu g\ ml^{-1}$) and 2.5% heat inactivated FCS. All incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

Dose-ranging study of rhIL-1β effects on cartilage

In order to characterize the biological response to rhIL-1β in both culture systems, samples were incubated for 48 h with or without rhIL-1β in the range of 0 to 2500 U ml^{-1} for chondrocytes in alginate beads and 0 to 30 U ml^{-1} for patellae and femoral head caps, respectively. Chondrocytes encapsulated in alginate beads were placed into DMEM/Ham's F12 culture medium containing 50 $\mu g\ ml^{-1}$ gentamycin, 2 mM L-glutamine, 0.25 $\mu g\ ml^{-1}$ amphotericin B, 2.5% heat inactivated FCS. Patellae and femoral head caps were incubated in RPMI Hepes 1640 medium supplemented with 2 mM L-glutamine, 40 $\mu g\ ml^{-1}$ gentamycin, 10 mM sodium pyruvate and 2.5% heat inactivated FCS. In all experiments, the medium was removed daily as described previously (van Beuningen *et al*., 1993).

Effects of NOS inhibitors and dexamethasone on IL-1-induced cartilage damage

Derived from the dose-ranging study, the drugs were tested in the presence of rhIL-1β at 25 U ml^{-1} for chondrocytes and 5 U ml^{-1} for cartilage samples. The five NOS inhibitors were used in the concentration range of 1 to 1000 μM. These products have shown different inhibitory profile towards NOS in various biological systems: N^{ω}-monomethyl-L-arginine (L-NMMA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), known to be active on constitutive and inducible NOS (Rees *et al*., 1990); aminoguanidine (AG), known to be more active on inducible NOS than on constitutive form; S-methylisothiourea (SMT), known to be selective of inducible NOS in cultured macrophages (Southan *et al*., 1995) and S-aminoethyl-isothiourea (AETU) recently shown to be more selective of inducible NOS of cartilage (Jang *et al*., 1996). Dexamethasone (0.1 to 100 μM) was tested in similar experimental conditions in order to assess the effect of corticosteroids in both systems. Chondrocytes encapsulated in alginate beads as patellae and femoral head caps were incubated for 48 h with rhIL-1β and NOS inhibitors or dexamethasone, with a daily change of culture medium.

The effects of the D-enantiomers, D-NMMA and D-NAME (1000 μM), which are inactive on NOS and the reversibility of NOS inhibition by their natural substrate, L-arginine (5000 μM), were also studied.

Chondrocytes proteoglycan synthesis

At the end of incubation (48 h), chondrocytes encapsulated in alginate beads were incubated in DMEM/Ham's F12 culture medium supplemented with L-glutamine (2 mM), gentamycin (50 $\mu g\ ml^{-1}$), amphotericin B (0.25 $\mu g\ ml^{-1}$), heat inactivated FCS (2.5%), ascorbic acid (25 $\mu g\ ml^{-1}$) and 10 $\mu Ci\ ml^{-1}$ of sodium sulphate [$Na_2{}^{35}SO_4$] for 4 h at 37°C in a 5% CO_2 atmosphere. Alginate beads were washed five times with 0.15 M NaCl and solubilized in citrate-EDTA buffer (55 mM/20 mM). Cells were digested overnight at 60°C in papain buffer [NaH_2PO_4 (0.2 M), EDTA (0.01 M), cysteine (0.01 M) and papain (6.75 U ml^{-1})]. The [^{35}S]-proteoglycan content was measured by liquid scintillation counting (Ultima Gold) with a LKB 1214 counter (Wallac, France).

Cartilage proteoglycan synthesis

At the end of incubation (48 h), cartilage samples were pulsed in RMPI Hepes 1640 medium supplemented with L-glutamine (2 mM), streptomycin (100 $\mu g\ ml^{-1}$), penicillin (100 ui ml^{-1}) and 0.6 $\mu Ci\ ml^{-1}$ of [$Na_2{}^{35}SO_4$] for 3 h at 37°C in a humidified 5% CO_2 atmosphere. After five washes in saline, cartilage entities were fixed overnight at room temperature in 0.5% cetylpyridinium chloride in formalin (10%). Thereafter, each sample was solubilized in counting tubes containing 0.5 ml of Soluene 350. The ^{35}S content of each patella and femoral head cap was measured by liquid scintillation counting (Hionic Fluor) as described above.

Nitrite assay

NO production was determined spectrophotometrically by measuring the accumulation of nitrite (NO_2^-) in culture supernatants by the Griess reaction (Green *et al*., 1982). Briefly, 100 μl of culture supernatant were mixed with 100 μl of Griess reagent (sulfanilamide [1%] in 2.5% of H_3PO_4 and N-Naphtylethylenediamine dihydrochloride [0.1%] in H_2O) for 5 min in 96-well plates. The optical density (OD_{550}) was measured by using a MR5000 (Dynatech) microplate reader. Nitrite concentrations were calculated with a standard curve of sodium nitrite ranging from 5 to 50 μM. Nitrite production was assessed as the cumulated nitrite release during the first and second day of incubation.

Cell viability

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Cells or cartilage entities were incubated with MTT (1 mg ml^{-1}) in DMEM/Ham's F12 and in RMPI Hepes 1640 for 4 h at 37°C respectively. Culture medium was removed by aspiration. The viability of cartilage entities was evaluated by confirmation that the cartilage entities were dark blue. After solubilization of alginate gel in a solution of sodium citrate and EDTA, chondrocytes viability was assessed by lysing cells in SDS–DMF buffer (20% w/v of SDS in 50% aqueous solution of DMF, pH 4.7) for 24 h at 37°C. The production of formazan within cells was quantified by measurement of absorbance at 550 nm on a MR5000 (Dynatech, France) microplate reader. Lactate dehydrogenase (LDH) activity, an indicator of drug cytotoxicity, was determined in culture supernatants of chondrocytes encapsulated in alginate beads by using a commercially available colorimetric assay kit (LK-100, Oxford Biochemical Research Inc. Michigan, U.S.A.). The rate of change in optical absorbance was measured at 490 nm on a MR5000 (Dynatech, France) microplate reader.

Statistics

Data are expressed as mean $\pm$ s.d. Each value is the mean of at least four samples. For proteoglycan synthesis, data are

expressed as percentage of variation of radiolabelled sodium sulphate incorporation *versus* healthy controls. For nitrite production, data are expressed as percentage of cumulated nitrite levels *versus* rhIL-1β controls. The results were analysed by ANOVA followed by a Fisher test or a Scheffe test. Differences with $P<0.05$ were considered as significant.

Materials

RPMI Hepes 1640 medium, Dulbecco's Modified Eagle Medium/Nut Mix F12 (DMEM/Ham's F12), L-glutamine, gentamycin, amphotericin B were obtained from Gibco BRL (Cergy Pontoise, France). Sodium pyruvate, penicillin, streptomycin, L-arginine, N^{ω}-monomethyl-L-arginine (L-NMMA), N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-monomethyl-D-arginine (D-NMMA), N^{ω}-nitro-D-arginine methyl ester (D-NAME), aminoguanidine (AG), S-aminoethyl-isothiourea (AETU), dexamethasone, N-naphtylethylenediamine dihydrochloride, sulfanilamide, cetylpyridinium chloride, pronase, and alginate were purchased from Sigma (St. Quentin Fallavier, France). S-methylisothiourea (SMT) was obtained from Calbiochem (Meudon, France). [$Na_2{}^{35}SO_4$] sodium sulphate (specific activity 100 mCi mmol^{-1}) was purchased from Amersham (Les Ulis, France). Soluene 350, Hionic Fluor and Ultima Gold were obtained from Packard (Rungis, France). Recombinant human interleukin-1β was a generous gift from Rhône-Poulenc-Rorer (Vitry sur Seine, France).

Results

Effects of rhIL-1β and NOS inhibitors on chondrocytes encapsulated in alginate beads

Chondrocytes maintained in culture for 2 days released detectable amounts of nitrite (Figure 1a). Addition of rhIL-1β induced a concentration-dependent production of nitrite reaching a maximal increase of 80% for 2500 U ml^{-1}. There was a corresponding concentration-dependent inhibition of radiolabelled sodium sulphate incorporation. However, for the highest concentrations of rhIL-1β proteoglycan synthesis inhibition plateaued (-37% to -3%) whereas nitrite release still increased (Figure 1a). The concentration of 25 U ml^{-1} of IL-1β was chosen to test the ability of NOS inhibitors to reverse the effects of IL-1β on chondrocytes, because it was in the middle range of the concentration-response curve for both parameters.

L-NMMA reversed the inhibitory effect of rhIL-1β on proteoglycan synthesis by 78% at 10 μM and overbalanced it at higher concentrations (Figure 1b). L-NAME corrected also the IL-1-induced suppression of proteoglycan synthesis, in a concentration-dependent manner, with an overbalancing effect at 1000 μM (Figure 1b). L-NMMA and L-NAME concomitantly reduced the release of nitrite in a concentration-dependent manner, with a maximal inhibitory effect of 80% and 65% respectively (Figure 1c). AG had no significant effect on the suppression of proteoglycan synthesis by IL-1β whatever the concentration used (Figure 1b). However, it reduced nitrite production in a concentration-dependent fashion from 7% at 1 μM to 88% at 1000 μM (Figure 1c). SMT was able to counteract the inhibitory effect of IL-1β on proteoglycan synthesis at 1 μM (105%), whereas it worsened it by 3.6-fold at 1000 μM (Figure 1b). At the same time, SMT concentration-dependently inhibited the release of nitrite (Figure 1c). It is noticeable that at the highest concentration, SMT induced a slight increase (10%) in LDH activity in culture medium, suggesting a possible toxic effect. AETU exacerbated the pejorative effect of IL-1 on proteoglycan synthesis from 1 μM (Figure 1b), whereas it inhibited nitrite release in a concentration-dependent manner (Figure 1c). At the highest concentrations (100 and 1000 μM), AETU increased significantly LDH activity in culture supernatant by 30% and 110% respectively whereas mitochondrial dehydrogenase activity was reduced by 10% and 59% respectively, showing a toxic effect. No significant changes were observed at the lowest concentrations of AETU, nor with L-NMMA, L-NAME or AG at all concentrations tested.

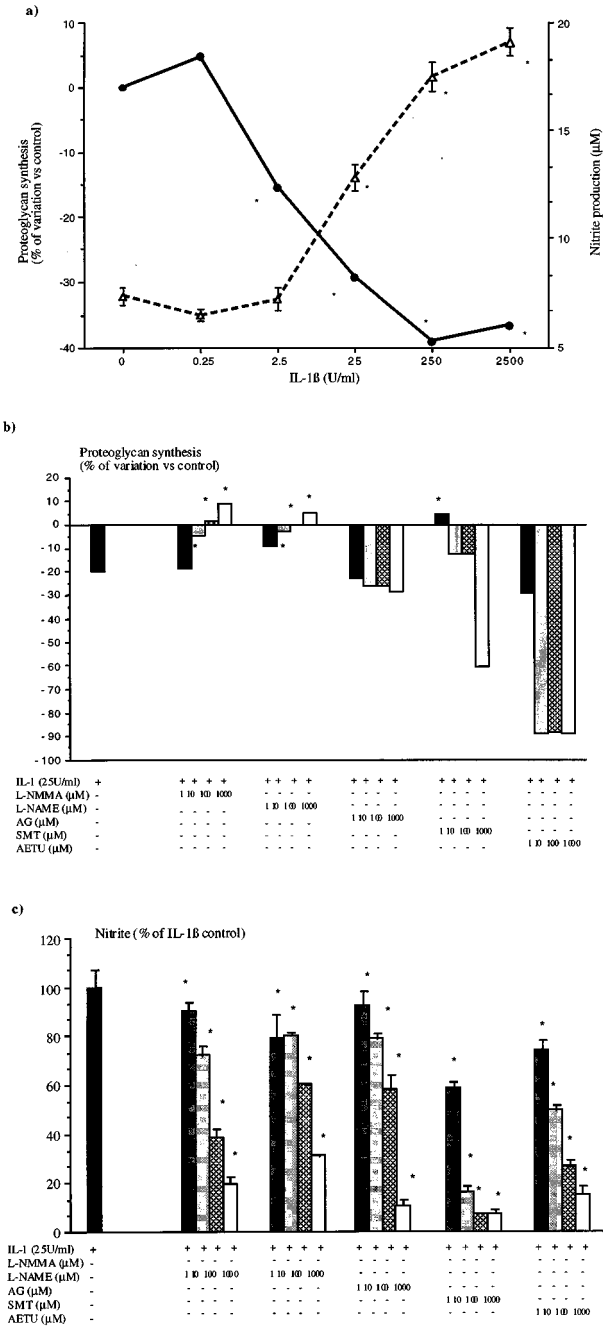

Figure 1 Effects of rhIL-1β and NOS inhibitors on chondrocytes encapsulated in alginate beads. Dose-ranging effect of rhIL-1β on proteoglycan synthesis (—●—) and nitrite release (- -△- -) (a). Effects of different NOS inhibitors on proteoglycan synthesis inhibition (b) and stimulated nitrite release (c). Data are mean$\pm$s.d. (a, c) or means percentage (b) ($n=4$ to 6). *$P<0.05$.

Effects of rhIL-1β and NOS inhibitors on patellae and femoral head caps

Patellae and femoral head caps maintained in culture for 2 days released detectable amounts of nitrite (Figures 2a and 3a). In patellae, rhIL-1β induced a decrease in proteoglycan synthesis and a large increase in nitrite accumulation which were both concentration-dependent up to 15 U ml^{-1} (Figure 2a). At this concentration of IL-1β, variation of proteoglycan synthesis reached -47% and nitrite level increased by twofold (Figure 2a). For a higher concentration (30 U ml^{-1} of IL-1β), incorporation of [$Na_2{}^{35}SO_4$] plateaued whereas production of nitrite still increased (2.5-fold). A similar pattern of results was obtained with femoral head caps, although the effects of IL-1β were quantitatively weaker than for patellae (Figure 3a). A concentration of 5 U ml^{-1} of IL-1β was chosen to investigate the ability of NOS inhibitors to reverse the effect of IL-1β on cartilage anabolism.

In patellae, L-NMMA partly reversed the inhibitory effect of IL-1β on proteoglycan synthesis from 10 μM (60%) to 1000 μM (87%) (Figure 2b) and reduced nitrite level from 100 μM (Figure 2c). In similar conditions, L-NAME corrected

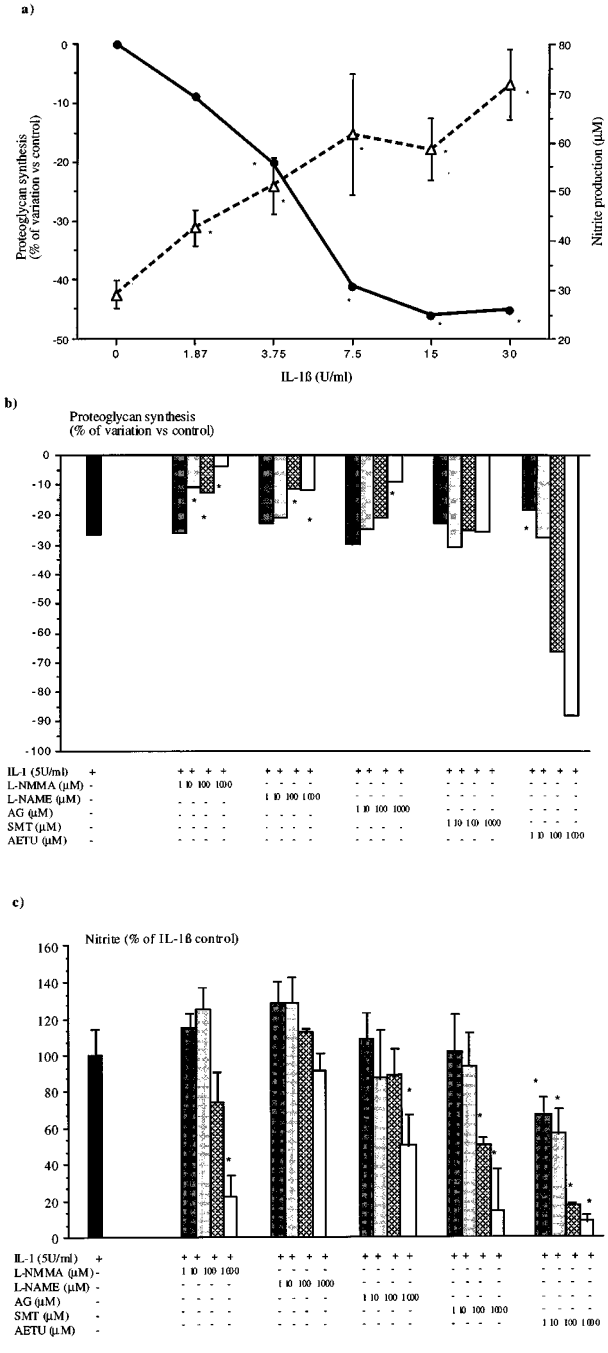

Figure 2 Effects of rhIL-1β and NOS inhibitors on patellae. Dose-ranging effect of rhIL-1β on proteoglycan synthesis (—●—) and nitrite release (- -△- -) (a). Effects of different NOS inhibitors on proteoglycan synthesis inhibition (b) and stimulated nitrite release (c). Data are mean $\pm$ s.d. (a, c) or means percentage (b) ($n=4$ to 6) *$P<0.05$.

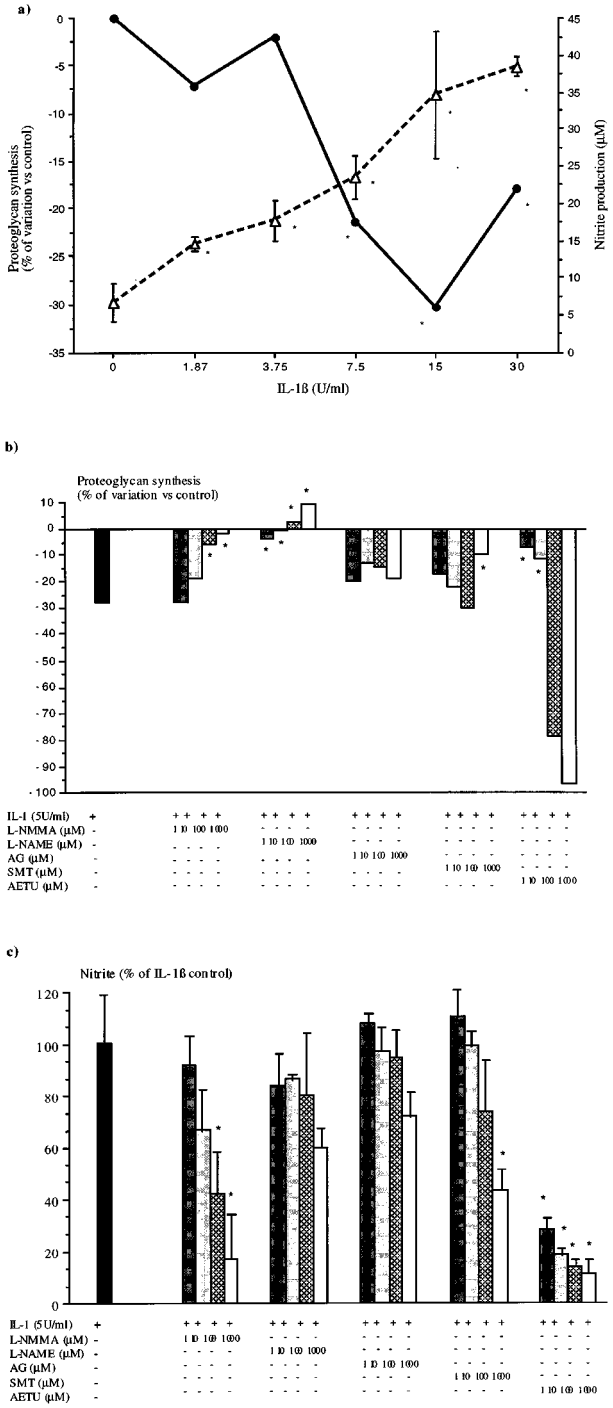

Figure 3 Effects of rhIL-1β and NOS inhibitors on femoral head caps. Dose-ranging effect of rhIL-1β on proteoglycan synthesis (—●—) and nitrite release (- -△- -) (a) Effects of different NOS inhibitors on proteoglycan synthesis inhibition (b) and stimulated nitric release (c). Data are mean $\pm$ s.d. (a, c) or means percentage (a) ($n=4-6$). * $P<0.05$.

proteoglycan synthesis by 57% at 100 μM and 59% at 1000 μM (Figure 2b), but had no significant effect on nitrite level (Figure 2c) AG was only effective at 1000 μM and corrected the inhibitory effect of IL-1β on proteoglycan synthesis by 68% (Figure 2b) when reducing its stimulatory effect on nitrite level by 66% (Figure 2c). In our experimental conditions, SMT was not active on proteoglycan synthesis (Figure 2b) but it reduced significantly nitrite levels at a concentration of 100 μM (Figure 2c). AETU slightly reversed the inhibitory effect of IL-1β on proteoglycan synthesis at 1 μM (32%), but exacerbated this effect at the highest concentrations (Figure 2b). Concomitantly, AETU reduced nitrite production in a concentration-dependent manner (Figure 2c), although we were not able to demonstrate if high concentrations of AETU were cytotoxic (all cartilage samples were dark blue in the MTT assay).

In femoral head caps, L-NMMA corrected the inhibitory effect of IL-1β on proteoglycan synthesis from 100 μM (78%) (Figure 3b) and concomitantly reduced nitrite level (55%) (Figure 3c). L-NAME corrected proteoglycan synthesis from 1 μM (96%) and overbalanced the effect of IL-1β over 100 μM (Figure 3b). At the same time, L-NAME had no significant effect on nitrite levels (Figure 3c). AG had neither any significant effect on inhibition of cartilage anabolism (Figure 3b) nor on stimulated nitrite level (Figure 3c). SMT was only effective on proteoglycan synthesis at 1000 μM (66%) (Figure 3b) when reducing nitrite level by 54% (Figure 3c). AETU partially corrected cartilage anabolism at 1 μM (74%) and at 10 μM (57%) but worsened IL-1β effect at higher concentrations (Figure 3b). AETU reduced mitrite release at all concentrations (Figure 3c).

Relevance of L-NMMA effects to the L-arginine: NO pathway

D-enantiomers, at the concentration tested (1000 μM), had no correcting effect on the suppression of proteoglycan synthesis induced by IL-1β in all systems, and they even worsened the IL-1 effect on femoral head caps. Concomitantly, the stimulatory effect of IL-1β on nitrite level was not significantly modified (Table 1).

The addition of L-arginine (5000 μM) to culture medium reduced the correcting effect of L-NMMA on proteoglycan synthesis by 7% in patellae and 5% in femoral head caps respectively, and significantly reduced it in chondrocytes (43%) (Table 1). L-arginine had a general tendency to reverse the inhibitory effect of L-NMMA on nitrite levels. This effect was less marked in patellae and femoral head caps than in chondrocytes. This might be due to differences in L-arginine concentration between culture medium since cartilage samples were cultured in RPMI Hepes 1640 medium containing a higher concentration of L-arginine (0.95 mM) than DMEM/Ham's F12 medium (0.7 mM). Such an explanation is further supported by the effect of L-arginine alone which significantly reduced nitrite levels in control cartilage samples when it increased it in embedded chondrocytes. No modification of proteoglycan synthesis was noted in these conditions. Taken together, these data suggest that the partial correcting effect of L-NMMA on IL-1-induced inhibition of proteoglycan synthesis is somewhat NO-related.

Effects of dexamethasone

When added in culture medium, dexamethasone had an inhibitory effect on proteoglycan synthesis by normal cartilage and chondrocytes (data not shown). In samples treated with IL-1β, no correcting effect was observed and, in contrast, dexamethasone worsened the inhibitory effect of IL-1β in a somewhat concentration-dependent manner (Figure 4a). However, this experiment showed that dexamethasone had a different pattern of effect on nitrite levels, depending on the system used (Figure 4b). Thus, dexamethasone reduced nitrite level in patellae by 50% whatever the concentration used. In contrast, no effect or even a stimulating effect was observed with chondrocytes and femoral head caps respectively. These data suggest that the inhibitory effect of dexamethasone in patellae might depend, at least in part, on the subchondral osseous tissue.

Discussion

In the present work, we compared the potency of new and classical NOS inhibitors on the inhibitory effect of rhIL-1β on rat cartilage anabolism by using three different culture systems: (1) isolated chondrocytes encapsulated in alginate beads; (2) patellae (cartilage and subchondral bone) and (3) femoral head

Table 1 Involvement of the L-arginine: NO pathway in the effect of L-arginine analog inhibitors on proteoglycan synthesis

	Patellae		*Femoral Head Caps*		*Chondrocytes*	
	Proteoglycan synthesis (% of control)	*Nitrite production* (% of IL-1β control)	*Proteoglycan synthesis* (% of control)	*Nitrite production* (% of IL-1β control)	*Proteoglycan synthesis* (% of control)	*Nitrite production* (% of IL-1β control)
Controls	100 ± 4.34	42.29 ± 2.67*	100 ± 1.50	35.36 ± 7.61*	100 ± 8.26	56.65 ± 3.13*
IL-1	77.54 ± 4.61*	100 ± 8.31	83.03 ± 6.54*	100 ± 16.86	79.25 ± 4.02*	100 ± 14.18
IL-1 + L-NMMA	96.5 ± 11.9	21 ± 12.2*	98.3 ± 10	17.1 ± 3.2*	109 ± 3	19 ± 2*
IL-1 + L-NAME	88.3 ± 7.2	91.7 ± 9.3	109.4 ± 2.5	59.6 ± 7.58*	105 ± 4	20.3 ± 4*
IL-1 + D-NMMA	80.85 ± 5.88*	91.31 ± 7.67	64.22 ± 21.42*	94.49 ± 8.19	78.38 ± 6.04*	83.84 ± 2.54
IL-1 + D-NAME	84.27 ± 7.13*	87.07 ± 5.72	55.86 ± 12.28*	92.93 ± 4.21	85.38 ± 3.01*	89.80 ± 5.13
IL-1 + L-NMMA	96.5 ± 11.9	21 ± 12.2*	98.3 ± 10	17.1 ± 3.2*	109 ± 3	19 ± 2*
IL-1 + L-NMMA + L-Arg	89.37 ± 7.26	29.51 ± 3.65*	92.85 ± 6.34	27.51 ± 10.77*	66.89 ± 0.74*	99.73 ± 6.63
L-Arg	89.19 ± 5.55	36.89 ± 4.10*	109.12 ± 5.09	25.92 ± 6.85*	99.02 ± 4.05	82.94 ± 6.41
IL-1 + L-Arg	73.38 ± 4.19*	70.13 ± 16.88*	94.56 ± 4.83	85.47 ± 12.75	86.85 ± 1.44*	93.73 ± 2.52

Chondrocytes or cartilage samples were incubated in the presence or absence of 25 U ml^{-1} and 5 U ml^{-1} Interleukin-1β (IL-1β) respectively for 2 × 24 h with either N$^{\omega}$-monomethyl-L-arginine (L-NMMA, 1000 μM); N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 1000 μM); N$^{\omega}$-monomethyl-D-arginine (D-NMMA, 1000 μM); N$^{\omega}$-nitro-D-arginine methyl ester (D-NAME, 1000 μM); L-arginine (L-Arg, 5000 μM). Proteoglycan synthesis is expressed as mean percentage relative to healthy controls. Nitrite production is expressed as mean percentage relative to IL-1 control ($n = 6$). *$P < 0.05$.

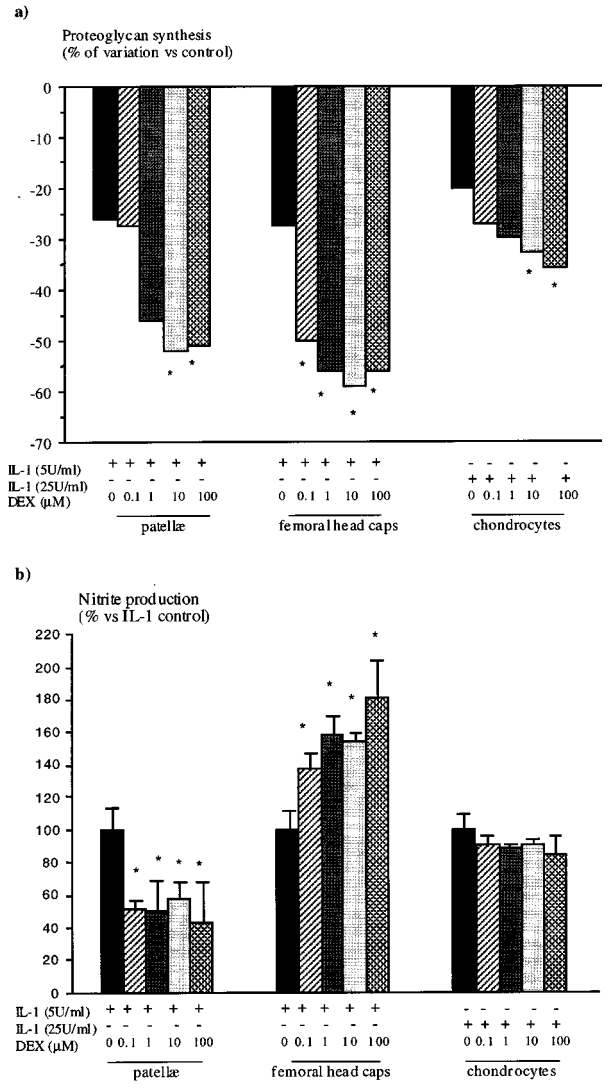

Figure 4 Effect of dexamethasone (DEX) on IL-1β effects on rat chondrocytes, patellae and femoral head caps; proteoglycan synthesis (a), nitrite release (b). ($n=6$). *$P<0.05$. Data are mean$\pm$s.d. (b) or means percentage ($n=6$). *$P<0.05$.

caps (cartilage) as intact anatomical entities. In the *ex vivo* culture systems, chondrocytes are in their original extracellular matrix when alginate beads may not totally reproduce the complexity of cell-matrix interactions. As a consequence, the comparison of biological responses in these systems, as for nitrite production and proteoglycan synthesis, are complementary for studying compounds with putative chondroprotective properties, such as NOS inhibitors.

In our experiment conditions, chondrocytes encapsulated in alginate beads and cartilage entities were incubated *in vitro* for 48 h in the presence of rhIL-1β. The incubation medium was refreshed daily to obtain an optimal response on both proteoglycan synthesis (van Beuningen *et al*., 1993) and nitrite production (Evans *et al*., 1996). Chondrocytes in alginate beads as cartilage entities responded concentration-dependently to rhIL-1β challenge by a large increase in nitrite levels and a marked suppression of proteoglycan synthesis, as previously observed in *in vitro* studies (Taskiran *et al*., 1994; Haüselmann *et al*., 1994; Järvinen *et al*., 1995). Interestingly, these biological responses dissociated from one another above 15 U ml^{-1} of IL-1β for cartilage samples and 250 U ml^{-1} for chondrocytes in alginate beads, since proteoglycan synthesis plateaued whereas nitrite release still increased. This suggests that both parameters had different concentration-response curves. However, it is worth noting that, in our hands, the different culture systems showed subtle functional differences. As regard to the IL-1β effect on proteoglycan synthesis, cartilage entities were much more sensitive than chondrocytes in alginate beads. Indeed, a concentration of 5 U ml^{-1} of IL-1β was sufficient to inhibit proteoglycan synthesis by -27% in patellae and -28% in femoral head caps, whereas a concentration of 25 U ml^{-1} of rhIL-1β was needed to obtain an inhibition of only -20% in chondrocytes. The exquisite sensitivity of cartilage samples to IL-1 exposure might be supported by differences in cell number but also by the influence of the peri-cellular matrix. Thus, it has been suggested that the signal transduction of IL-1 in chondrocytes might be regulated by cell-extracellular matrix interactions, as the $\alpha 5\beta 1$-fibronectin complex, through the formation of specialized adhesive membrane domain (focal adhesion) (Luo *et al*., 1997). These structural links between extracellular matrix and cytoskeleton are also able to control constitutive and growth factor-stimulated proteoglycan synthesis in chondrocytes and may be disrupted by NO overload (Clancy *et al*., 1997). It remains to be established whether chondrocytes cultured in alginate beads for several months and/or in the presence of fibronectin will be more susceptible to IL-1 challenge, but our data emphasize that *ex vivo* cultures systems may be the most predictive of joint pathological situations.

Another interesting finding was that, at the same low concentration of IL-1, inhibition of proteoglycan synthesis was more marked in patellae than in femoral head caps. One plausible explanation is that the subchondral bone may interfere and modify cartilage metabolism in patellae. Indeed, it has recently been shown that the release of glycosaminoglycan from cartilage was increased when the latter was incubated with bone cells (Westacott *et al*., 1997). As osseous cells can produce NO after exposure to cytokines or endotoxins (Collin-Osdoby *et al*., 1995), bone-derived NO may modify proteoglycan synthesis in cartilage. This is consistent with higher levels of nitrite produced by patellae than by femoral head caps. In addition, cartilage depth varies between these two cartilage samples and as NO production differs significantly between superficial and deep layers (Fukuda *et al*., 1995a), this may contribute to differences between our systems.

From a pharmacological point of view, evidence has accumulated that chondrocyte inducible NOS plays an important role in the pathogenesis of arthritis, leading to the notion that NO inhibition could have anti-rheumatic effects (Taskiran *et al*., 1994). In our experiments, all NOS inhibitors were able to reduce nitrite production by chondrocytes encapsulated in alginate beads, in a concentration-dependent manner. In this system, S-substituted isothioureas were the most potent, but some cytotoxicity was observed at the highest concentrations at AETU. In organ culture systems, NOS inhibitors were able to reduce significantly the stimulatory effect of IL-1β on nitrite production only at the high concentration (1000 μM), with some exception for aminoguanidine in femoral head caps and L-NAME on both samples. These results confirm the weak inhibitory potency of L-NAME on inducible NOS isoform (Stefanovic-Racic *et al*., 1995; Jang *et al*., 1996) and the need for a high concentration of aminoguanidine to obtain some correcting effect against IL-1 (Järvinen *et al*., 1995; Blanco *et al*., 1995; Murrell *et al*., 1996). L-NMMA inhibited nitrite production in both cartilage samples, as recently reported for cytokine-stimulated chon-

drocytes (Stefanovic-Racic *et al*., 1995; Jang *et al*., 1996). In the same experimental conditions, SMT was quite as effective as L-NMMA (Jang *et al*., 1996) when AETU was the most powerful inhibitor, although with possible cytotoxicity at high concentrations. Taken together, our results show that: (i) the ability of NOS inhibitors to reduce nitrite level decreases from chondrocytes to cartilage samples except for L-NMMA and AETU, a phenomenon possibly attributable to a limited diffusion of compounds in cartilage matrix and (ii) NOS inhibitors can be roughly classified as the following rank order of potency: AETU > L-NMMA ⩾ SMT > AG ⩾ L-NAME which is close to that reported for bovine isolated chondrocytes (Jang *et al*., 1996).

When considering the inhibitory effect of IL-1β on cartilage anabolism, a variable correcting effect of NOS inhibitors was observed depending on both molecules and tissues concerned. In chondrocytes encapsulated in alginate beads, L-NMMA and L-NAME were the most effective since they were able to reverse and even to overbalance the inhibitory effect of IL-1β on proteoglycan synthesis. We report for the first time that NOS inhibitors with selectivity for inducible isoform were ineffective. This may not result from a rate limited diffusion of drugs in chondrocytes, as suggested for AG (Evans *et al*., 1996), since we observed a significant reduction of nitrite levels. In organ culture systems, L-NMMA and L-NAME were also the most effective on proteoglycan synthesis. As their D-isomers, which were unable to correct nitrite levels, also lacked any correcting effect on the inhibition of proteoglycan synthesis, this chondroprotective effect is likely not due to a fully non specific activity, as sometimes reported for L-arginine analogs (Peterson *et al*., 1992). In addition, L-arginine tended to reverse the correcting effect of L-NMMA on proteoglycan synthesis, further suggesting that it was, at least in part, NO-related (Stadler *et al*., 1991; Taskiran *et al*., 1994). Taken together, our results show that NOS inhibitors: (i) had a more marked correcting (or worsening for AETU) effect on proteoglycan synthesis in chondrocytes than in cartilage samples and (ii) can be roughly classified as the following rank order of potency: L-NAME ⩾ L-NMMA > > AG > SMT > > AETU.

Based on the data mentioned above for L-NMMA, it should be argued that NO modulation may be useful for correcting the effect of IL-1 on cartilage anabolism. However, a more detailed analysis suggests that there is no general negative correlation between nitrite levels and proteoglycan synthesis, particularly in cartilage samples. Indeed, L-NAME corrected proteoglycan synthesis at concentrations which were ineffective on nitrite production. SMT had a weak effect on proteoglycan synthesis inhibition although it was very effective in reducing nitrite levels in patellae. These results demonstrate that the chondroprotective potency of NOS inhibitors can not be extrapolated from their effects on NO production by joint-derived cells.

Our results suggest that in a pathological situation, NO modulation may not be sufficient *per se* to restore cartilage anabolism and that the relationships between NO release and inhibition of proteoglycan synthesis by IL-1 are complex. Several hypotheses may explain such a complexity. Firstly, recent studies have suggested that the ability of NO to reproduce the disrupting effects of IL-1 on the regulation of proteoglycan synthesis is by a focal adhesion-dependent system (Clancy *et al*., 1997). However, we do not know presently whether some signalling pathways as F-actin/RhoA are firmly NO-dependent or whether these are unequally affected by NOS inhibitors (Clancy *et al*., 1997). Secondly, the effects of IL-1 on chondrocytes also include the generation of oxygen-derived radical species (Rathakaishnan *et al*., 1992) and the activation of heme-dependent protein systems, such as cyclo-oxygenases (Lyons-Giordano *et al*., 1993) and perhaps heme oxygenases (Willis *et al*., 1996). These systems can either interact with the L-Arg: NO pathway (Tsai, 1994) or combine directly with NO to generate new species (Beckman & Koppenol, 1996), thus contributing possibly to the modulation of proteoglycan synthesis (Fukuda *et al*., 1995b). As a consequence, the potency of NOS inhibitors towards rhIL-1β effects can be related to their potency for both L-Arg: NO pathway and the above mentioned systems. At least, amongst inducible NOS isoforms, cartilage iNOS may have some functional peculiarities. Although our results suggest a possible contribution of subchondral bone in patellae, we demonstrated that rat chondrocytes iNOS appears to be corticosteroid-resistant as reported for other species (Palmer *et al*., 1992; 1993). As a consequence, inhibitors with selectivity for macrophages iNOS may not systematically preserve their potency on chondrocytes.

Taken together, one may understand that the chondroprotective potencies of NOS inhibitors may differ significantly and can lead to a partial restoration of proteoglycan synthesis. Moreover, as IL-1 effects in cartilage may involve the NO pathway in different ways, a NOS inhibitor with a broad spectrum of action, such as L-NMMA, may be the most efficient.

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