INTRODUCTION
Nitric oxide (NO) has been identified as one of the factors responsible for the deterioration of cartilage in arthritis (1), however the mechanisms by which it causes matrix loss have not been clearly defined. Insulin-Like Growth Factor-I (IGF-I) supports the maintenance of healthy cartilage under normal conditions. However, chondrocytes in cartilage from arthritic joints respond poorly to IGF-I (2). Recent studies with iNOS knockout mice (3) suggest that NO is responsible for part of cartilage insensitivity to IGF-I. Cartilage from wild-type mice with antigen-induced arthritis did not respond to IGF-I, however cartilage from iNOS knockout mice did increase proteoglycan synthesis when exposed to IGF-I. The current studies were designed to characterize the relationship between NO and chondrocyte responsiveness to IGF-I in vitro, and to define the mechanism(s) by which NO alters IGF-I stimulation of chondrocyte proteoglycan synthesis.

METHODS
Lapine cartilage slices and primary monolayer cultures of articular chondrocytes (AC) were prepared from knee and shoulder joints and maintained as previously described (4). Some studies used cartilage slices removed from human knees secondary to arthroplasty for osteoarthritis. Tissues were exposed to NO from the NO donor S-Nitroso-N-acetylpenicillamine (SNAP), by activation with 2 ng/ml interleukin-1 (IL-1), or by transduction of an adenoviral vector for iNOS (Ad-iNOS). N\(^2\)-monomethyl-L-arginine (L-NMA) was used to inhibit NO synthesis by iNOS. NO production was estimated by measuring nitrite in conditioned media using a spectrophotometric assay based on the Griess reaction. Proteoglycan synthesis was measured as the incorporation of \(^{35}\)SO\(_4\)\(^{2-}\) into macromolecular material [8 hr pulse labeling with \(^{35}\)SO\(_4\)\(^{2-}\) from 24 to 32 hr after stimulation with IGF-I as previously described (4)]. Western analysis of IGF-I receptor phosphorylation was done using published procedures (5) using antibody to human IGF-IReta (Santa Cruz Biotechnology) and phosphotyrosine (PY20, Transduction Labs).

RESULTS
Addition of SNAP to cartilage cultures generated nitrite of 40-50 uM and decreased the ability of IGF-I to stimulate proteoglycan synthesis at all doses tested (5-100 ng/ml). For example, 50 ng/ml IGF-I increased proteoglycan \(^{35}\)SO\(_4\)\(^{2-}\) incorporation from 300 to 485 ± 33 cpm/10 mg cartilage in control slices; in slices exposed to SNAP, this increase was diminished by 50% (basal = 203 ± 15; 50 ng/ml IGF-I =297 ± 47 cpm/10 mg). Similar inhibition was seen when chondrocytes in monolayers were exposed to SNAP. Chondrocytes transduced with Ad-iNOS at 6 x 10\(^6\) pfu/well synthesized NO to yield nitrite of 24 uM/24 hr. This endogenously produced NO also inhibited IGF-I stimulated proteoglycan synthesis by over 70% (stimulated increase in proteoglycan synthesis was decreased from 724 to 195 cpm/well). The effects of NO were reversible; if L-NMA was added to inhibit NO synthesis by iNOS, IGF-I stimulated proteoglycan synthesis returned to normal within 48 hr.

Cartilage from arthritic patients generated NO at low, but significant levels. Thus experiments were done to 1) determine if the relations seen between NO and response to IGF-I in normal rabbit cartilage/chondrocytes could be seen in human material, and 2) to determine if culture with L-NMA to blunt chondrocyte NO production in these materials could affect the response to IGF-I. Cartilage removed from OA knees responded poorly to IGF-I (stimulated increase in proteoglycan synthesis was from a basal value of 90 to 140 cpm/10 mg); however, if the damaged cartilage was cultured for 5 days with 0.5 mM L-NMA to inhibit NO synthesis, the response to IGF-I was restored (increase from 100 to 350 cpm/10 mg). Comparable results have been obtained with cartilage from three patients.

NO synthesized by chondrocytes stimulated with IL-1 is sufficient to diminish the response to IGF-I. 50 ng/ml IGF-I increased AC proteoglycan synthesis from 1176 ± 72 to 3216 ± 172 cpm \(^{35}\)SO\(_4\) incorporated/well. This was decreased to 2304 ± 168 cpm if the cells were exposed to 2 ng/ml IL-1 for 6 hr before addition of IGF-I (NO = 13 ± 1 uM). If L-NMA was added to block NO synthesis (NO = 3 ± 1 uM), IGF-I stimulated proteoglycan synthesis was increased to 2904 ± 72 cpm.

Previous studies of chondrocytes from OA cartilage suggest that alterations in neither IGF-I receptor number nor receptor affinity for IGF-I are responsible for the observed insensitivity (6,7), thus NO mediated changes in signaling beyond these steps were evaluated. The Beta (intracellular) units of IGF-I receptors were immunoprecipitated from AC lysates at times from 1 to 20 min after stimulation with IGF-I. Proteins were separated by electrophoresis and blotted onto nitrocellulose membranes. The membranes were probed with antibody to phosphotyrosine. Significant phosphotyrosine was identified in bands migrating with the same molecular weight as the IGF-I receptor. If the chondrocytes had been exposed to NO generated by SNAP or synthesized by iNOS, IGF-I receptor autophosphorylation was inhibited.

DISCUSSION
These studies support the hypothesis that NO is responsible for part of the arthritic cartilage/chondrocyte insensitivity to the anabolic actions of IGF-I. These effects can be mimicked by exposure to exogenous NO from nitric oxide donors, and by transduction of normal cells with iNOS. The use of iNOS transfected cells provides a model which more closely mimics the in vivo production of NO, as the release of NO by donors such as SNAP is different from that generated by iNOS in magnitude, temporal pattern, and subcellular location with respect to potential effectors of the actions of NO on the cell. The effects of NO to inhibit IGF-I stimulation of chondrocyte proteoglycan synthesis are reversible. The specific mechanisms involved in the NO inhibition of IGF-I autophosphorylation are currently under study. The response to IGF-I can be restored in diseased cartilage by inhibition of NO synthesis with L-NMA, establishing a model of human material which can be used to: 1) confirm that findings in lapine material are relevant to those in humans; and 2) explore therapeutic modalities in vitro which may translate to in vivo treatment to arrest/slow the progress of cartilage degeneration in human disease.

REFERENCES

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