Nitric Oxide: an Important Articular Free Radical

GEORGE A. C. MURRELL, MARTIN M. DOLAN, DANIEL JANG, CSABA SZABO, RUSSELL F. WARREN and JO A. HANNAFIN


This information is current as of July 24, 2010

Reprints and Permissions  Click here to order reprints or request permission to use material from this article, or locate the article citation on jbjs.org and click on the [Reprints and Permissions] link.

Publisher Information  The Journal of Bone and Joint Surgery
20 Pickering Street, Needham, MA 02492-3157
www.jbjs.org
Nitric Oxide: an Important Articular Free Radical*

BY GEORGE A. C. MURRELL, M.B.B.S., D.PHIL.†, MARTIN M. DOLAN, B.A.‡, DANIEL JANG, B.SC.†,
CSABA SZABO, M.D., PH.D.§, RUSSELL F. WARREN, M.D.¶, AND JO A. HANNAFORD, M.D., PH.D.§, NEW YORK, N.Y.

Investigation performed at the Laboratory for Soft Tissue Research and Sports Medicine Service, The Hospital for Special Surgery, New York City

ABSTRACT: Nitric oxide is a small molecule that is synthesized by a family of enzymes, the nitric oxide synthases, and is overproduced in rheumatoid arthritis and osteoarthritis. The aim of this investigation was to elucidate the potential sources of nitric oxide in joint tissues and to determine if the production of nitric oxide could be inhibited by dexamethasone or methotrexate, two agents that inhibit other forms of inducible nitric oxide synthase. Methotrexate inhibits the synthesis of biopterin, which is a co-factor for nitric oxide synthase.

Explants of human and bovine cartilage and cultured chondrocytes released large amounts of nitrite, the stable end product of nitric oxide, when stimulated with endotoxin, interleukin-1β, or tumor necrosis factor-α. The production of nitrite was time-dependent and endotoxin, interleukin-1β, and tumor necrosis factor-α dose-dependent and was inhibited by the nitric-oxide-synthase inhibitors Nω-nitro-L-arginine methyl ester and aminoguanidine. The inducible nitric oxide synthase in bovine chondrocytes was calcium-dependent and was inhibited by high concentrations of methotrexate or dexamethasone. No constitutive nitric-oxide-synthase activity and little or no inducible nitric-oxide-synthase activity were demonstrable in explants or cell cultures derived from menisci. Fresh explants of bovine articular synovial tissue constitutively released nitrite that was inhibited by Nω-nitro-L-arginine methyl ester, but the release could not be enhanced by endotoxin, interleukin-1β, or tumor necrosis factor-α. There was no constitutive or inducible production of nitrite by explants or cells derived from the synovial tissue or shoulder capsule of a human or by explants or cells derived from canine anterior cruciate, posterior cruciate, medial collateral, lateral collateral, or patellar ligaments. Taken together, these results indicate that chondrocytes represent the major source of inducible nitric oxide synthase and nitric oxide during inflammation or infection of a joint.

CLINICAL RELEVANCE: Since nitric oxide is a free radical, its effects are extremely rapid, local, and potentially toxic. Induction of high levels of chondrocytic nitric oxide during infection or inflammation may be responsible in part for the damage to cartilage that occurs in some inflammatory arthropathies. Agents that inhibit nitric oxide synthase, especially selective inhibitors of the inducible form of nitric oxide synthase, may offer new and useful means of inhibiting the destruction of cartilage.

Nitric oxide is a small molecule synthesized from the amino acid L-arginine by a family of enzymes called the nitric oxide synthases. Nitric oxide is a multifunctional intercellular and intracellular messenger molecule that plays a role in a variety of physiological processes. For instance, nitric oxide synthesized by a constitutive isoform of nitric oxide synthase in the vascular endothelium is responsible for the regulation of blood pressure and the control of platelet aggregation and may regulate cardiac muscle contractility. In the central nervous system, nitric oxide is produced by a different constitutive isoform of nitric oxide synthase and is a neurotransmitter involved in memory and motor function. In the peripheral nervous system, nitric oxide is involved in the modulation of relaxation of the gastrointestinal tract, bladder, and corpus cavernosum. Nitric oxide is the final common mediator of erection.

Macrophages, peripheral-blood monocytes, neutrophils, and lymphocytes produce large amounts of nitric oxide by means of a distinct inducible isoform of nitric oxide synthase during host defense, immunological reactions, and septic shock. This inducible form of nitric oxide synthase can be activated by endotoxin (a cell-wall lipopolysaccharide of Escherichia coli) or by inflammatory mediators, such as interleukin-1β, tumor
necrosis factor-α, and interferon-δ, and it can be inhibited by dexamethasone\textsuperscript{16}. Increases in the concentrations of nitrite, the stable end product of nitric oxide, have been found in the synovial fluid and serum of patients who have rheumatoid arthritis or osteoarthritis\textsuperscript{5}. Inhibitors of nitric oxide synthases have reduced the severity of autoimmune arthritis in a murine spontaneous arthritis model\textsuperscript{19}, a murine streptococcal cell-wall-induced arthritis model\textsuperscript{16}, and an adjuvant-induced arthritis rat model\textsuperscript{16}.

The aim of the present investigation was to determine which joint tissues are sources of nitric oxide and to determine if the production of nitric oxide can be inhibited by dexamethasone and methotrexate.

**Materials and Methods**

**Materials**

Dulbecco modified Eagle medium, calcium and magnesium-free Dulbecco phosphate-buffered saline solution, Hanks solution, antibiotic-antimycotic solution (number 600-5240; 10,000 units of penicillin-G sodium per milliliter, 10,000 micrograms of streptomycin sulfate per milliliter, and twenty-five micrograms of amphotericin B per milliliter), Heps solution (238.3 grams per liter), trypsin solution (0.25 percent trypsin in Hanks solution), and fetal calf serum were purchased from Gibco Laboratories, New York, N.Y. Human recombinant tumor necrosis factor-α was a gift from Grace Wong, Genentech, South San Francisco, California. All other chemicals and biochemicals, including human recombinant interleukin-1β, aminoguanidine, and No-nitro-L-arginine methyl ester were purchased from Sigma Chemical, St. Louis, Missouri.

**Cartilage**

Cartilage was obtained from the knee of a seven-year-old girl who was having an arthrodesis of the knee for proximal femoral focal deficiency. Bovine occipital articular cartilage was obtained from the articular surfaces of the occipital condyle of freshly slaughtered calves. Bovine knee cartilage was obtained from the knee of a seven-year-old girl who was having an arthroscopic partial meniscectomy. Canine menisci were harvested from adult mongrel dogs that had been killed for other research purposes. Each sterile tissue sample was immediately placed in cold (4 degrees Celsius) tissue culture medium containing 90 percent Dulbecco modified Eagle medium, 1 percent antibiotic-antimycotic solution, and 10 percent fetal calf serum, pH 7.35. The cartilage was washed in normal sterile saline solution, 10 percent antibiotic-antimycotic solution in Hanks medium, and normal saline solution. Disks of cartilage (one millimeter thick and four millimeters in diameter) were cut with a biopsy punch (SMS Instruments, Columbia, Maryland), placed in twenty-four or ninety-six-well tissue culture plates in the presence of one milliliter or 250 microliters of medium without fetal calf serum, and cultured at 37 degrees Celsius in 5 percent carbon dioxide and 95 percent air. All explants were assayed on the day of procurement, with the exception of one set of explants of bovine occipital cartilage that was maintained in culture for three months with twice-weekly changes of the medium (Dulbecco modified Eagle medium and 10 percent fetal calf serum) before it was assayed for nitric-oxide-synthase activity.

Chondrocytes were obtained by collagenase digestion\textsuperscript{1} of slices of bovine occipital condyles in 0.025 percent collagenase, 1 percent antibiotic-antimycotic solution, and 2 percent Heps, with gentle agitation at 37 degrees Celsius for sixteen hours. The cell suspension was centrifuged at 2000 times gravity for fifteen minutes; the supernatant was discarded, and the cells were washed in 10 percent antibiotic-antimycotic solution and plated in seventy-five-milliliter culture flasks in thirty milliliters of medium per flask with fetal calf serum at 2 × 10\textsuperscript{6} cells per flask, culture conditions that support the maintenance of the chondrocyte phenotype\textsuperscript{5}. When the cells were confluent (usually after seven to ten days), they were trypsinized and seeded in ninety-six-well plates at 10\textsuperscript{4} cells per well (passage 1). The next morning, nitric oxide synthase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed, as will be described.

**Menisci**

Bovine menisci were obtained from the knee joints of freshly slaughtered calves and then were cut into one-millimeter-thick strips. The strips were fashioned into disks that were one millimeter thick and four millimeters in diameter. Care was taken to use the central portions of the menisci to avoid any overlying synovial tissue. Fresh disks were placed in ninety-six-well tissue culture plates. All explants were assayed on the day of procurement.

Free bovine meniscal cells were obtained by collagenase digestion, plated, and seeded as described for the cultured chondrocytes.

Human menisci were obtained from patients who were having an arthroscopic partial meniscectomy. Canine menisci were harvested from adult mongrel dogs that had been killed for other research purposes. Each sterile tissue sample was immediately placed in cold tissue culture medium and was dissected into one-millimeter cubes. Six cubes were placed into each well of a six-well tissue culture plate. Media were changed every three days throughout the culture period. When the explant outgrowths were confluent (usually after two weeks), the cell layers were trypsinized and transferred to large tissue culture flasks (passage 1). When the cells were confluent (usually after ten days), they were trypsinized and seeded at 10\textsuperscript{5} cells per well in ninety-six-well plates (passage 2) for all assays.

**Ligaments**

Explants and cell cultures derived from bovine and canine anterior cruciate, posterior cruciate, medial col-
lateral, and lateral collateral ligaments were examined under the same conditions as those for the menisci.

**Synovial Tissue**

Bovine synovial tissue was obtained from the knee joints of freshly slaughtered calves and was dissected into three by three-millimeter explants. One explant was placed in each well of a ninety-six-well tissue culture plate. All explants were assayed on the day of procurement.

Free cells were obtained from the bovine synovial tissue by collagenase digestion, plated, and seeded as described.

The shoulder capsule and synovial tissue were obtained from a twenty-one-year-old woman who was having a forequarter amputation for chondrosarcoma and were carefully separated and dissected into onemillimeter cubes. Cells were cultured from explant outgrowths under the same conditions as those for meniscal outgrowths.

**Determination of Constitutive and Inducible Nitric-Oxide-Synthase Activity**

The presence of constitutive or inducible nitric-oxide-synthase activity in explants or cultured cell lines was determined by replacing the medium in each well with fresh medium that did not contain fetal calf serum but did contain either (1) no additives; (2) one microgram of endotoxin per milliliter, 100 nanograms of tumor necrosis factor-α per milliliter, or ten nanograms of interleukin-1β per milliliter; (3) a nitric-oxide-synthase inhibitor (one milligram of No-nitro-L-arginine methyl ester per milliliter or five-millimolar aminoguanidine); or (4) an inducible nitric-oxide-synthase inducer and a nitric-oxide-synthase inhibitor. There were six replicates in each group. As evaporation of the medium can falsely elevate nitrite concentrations, blank plates containing the same media under the same conditions but no cells or explants were used to control for evaporation. The plates were cultured for twenty-four hours (with the exception of the time-response assays, which were cultured for as long as ninety-six hours). One hundred microliters of medium was removed from each well and was transferred to a ninety-six-well plate for nitrite analysis. The amount of any nitrite detected in the blank plate was subtracted from the amount detected on the experimental plate. Constitutive nitric-oxide-synthase and inducible nitric-oxide-synthase activities could be determined with the following equations. Constitutive nitric-oxide-synthase activity = no additives – nitric-oxide-synthase inhibitor. Inducible nitric-oxide-synthase activity = (nitric-oxide-synthase inducer – [nitric-oxide-synthase inducer + nitric-oxide-synthase inhibitor]) – constitutive nitric-oxide-synthase activity. Viability of the cell layers or of the explants was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion to formizan, as will be described. Data were reported only when there was no inhibition of mitochondrial 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide conversion to formizan.

**Nitrite Assay**

Nitrite, a stable end product of nitric oxide, was measured in the media of cultured cells and explants with use of a spectrophotometric method based on the Greiss reaction. One hundred microliters of Greiss reagent (1 per cent sulfanilamide and 0.1 per cent naphthyl ethylenediamine in 5 per cent phosphoric acid) was added to 100-microliter samples of cell culture medium. The optical density was measured at 550 nanometers (reference filter, 650 nanometers) with a 340 ATTC microplate photometer (Tecan US, Research Triangle Park, North Carolina). Nitrite concentrations were calculated by comparison with the optical density of standard solutions of sodium nitrite prepared in Dulbecco modified Eagle medium.

**Cell Viability**

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formizan. Cells or explants in ninety-six-well plates were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.4 milligram per milliliter) for sixty minutes. Culture medium was removed by aspiration. The viability of the explants was grossly evaluated by confirmation that the explants were dark blue. (Viable tissue stains dark blue and non-viable tissue remains unstained.) Cell monolayer viability was assessed by solubilizing of the cells in 200 microliters of dimethyl sulfoxide. The extent of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formizan within cells was quantified by measurement of absorbance at 550 nanometers (reference filter, 650 nanometers). The production of formizan was expressed as the average percentage of that observed in the control group.

**Morphological Analysis**

The morphological characteristics of cultured cell lines were evaluated directly and from photographs with use of an Olympus BHS inverted microscope and C35AD4 camera (Olympus, Tokyo, Japan).

**Statistical Analysis**

Statistical analysis was performed with use of unpaired and paired (for the time-response) two-tailed Student t tests.

**Results**

**Morphological Analysis of Cell Monolayers**

Morphological analyses confirmed that all cell lines derived from the collagenase-digested bovine articular free radical.
lary cartilage displayed the same rounded, cobble-stone appearance characteristic of chondrocytes during the short-term (passage-1) culture conditions of these experiments. The morphological appearance of the cell lines derived from explants of human meniscal cells was between that of chondrocytes and that of fibroblasts—that is, they were neither round nor the long cellular processes of fibroblasts. The cell lines derived from human synovial tissue were large, round, and flattened, and those derived from the human shoulder capsule were more fibroblastic in appearance. The cell lines derived from canine anterior cruciate, posterior cruciate, medial collateral, lateral collateral, and patellar ligaments had the appearance of fibroblasts.

**Nitric-Oxide-Synthase Activity in Explants of Cartilage and Cultured Chondrocytes**

There was no constitutive nitric-oxide-synthase activity in the explants of articular cartilage from the human knee or in the bovine articular cartilage (Table I). When stimulated with endotoxin, the explants of articular cartilage from the human knee released signifi-

---

**TABLE I**

**Nitric-Oxide-Synthase Activity in Explants***

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Constitutive</th>
<th>1 µg/ml Endotoxin</th>
<th>10 ng/ml Interleukin-1β</th>
<th>100 ng/ml Tumor Necrosis Factor-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human knee cartilage</td>
<td>&lt;1</td>
<td>588 ± 300†</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>Bovine cartilage</td>
<td>1</td>
<td>163 ± 40†</td>
<td>68 ± 15‡</td>
<td>34 ± 6†</td>
</tr>
<tr>
<td>Occipital</td>
<td>Fresh</td>
<td>1</td>
<td>64 ± 20‡</td>
<td>48 ± 15‡</td>
</tr>
<tr>
<td></td>
<td>3 mos. in culture</td>
<td></td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>Bovine meniscus</td>
<td>&lt;1</td>
<td></td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Bovine synovial tissue</td>
<td>7 ± 1</td>
<td>7 ± 1</td>
<td>4 ± 2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Bovine ligament</td>
<td>Anterior cruciate</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Posterior cruciate</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Medial collateral</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Lateral collateral</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*The values are given as the mean and the standard error of the mean number of nanomoles of nitrite per gram of wet weight after twenty-four hours. There were six replicates in each group. No activity was detected in the presence of one-millimolar aminoguanidine or one milligram of No-nitro-L-arginine methyl ester per milliliter.

†P < 0.001, compared with the control (unpaired two-way Student t test).

‡P < 0.01, compared with the control (unpaired two-way Student t test).

---

**FIG. 1**

Graph showing the mean (and standard error of the mean) inducible nitric-oxide-synthase activity in fresh explants of cultured bovine occipital cartilage (four millimeters in diameter). Induction of nitrite (NO₂⁻) occurred in a dose-response manner when cartilage was exposed to endotoxin (End), human recombinant tumor necrosis factor-α (TNF-α), or human recombinant interleukin-1β (IL-1β). In each case, the production of nitrite was inhibited by 0.5-millimolar aminoguanidine (+), an inhibitor of nitric oxide synthase. There were six replicates in each group. * = p < 0.05, ** = p < 0.01, and *** = p < 0.001, compared with the control (unpaired two-way Student t test).
TABLE II
NITRIC-OXIDE-SYNTHASE ACTIVITY IN BOVINE TISSUES*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Constitutive</th>
<th>1 μg/ml Endotoxin</th>
<th>10 ng/ml Interleukin-1β</th>
<th>100 ng/ml Tumor Necrosis Factor-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occipital cartilage</td>
<td>3 ± 1</td>
<td>91 ± 8*</td>
<td>69 ± 2†</td>
<td>9 ± 2†</td>
</tr>
<tr>
<td>Meniscus</td>
<td>&lt;1</td>
<td>3 ± 1‡</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Synovial tissue</td>
<td>&lt;1</td>
<td></td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*The values are given as the mean and the standard error of the mean number of nanomoles of nitrite per gram of wet weight after twenty-four hours. There were six replicates in each group. No activity was detected in the presence of one milligram of Na-nitro-L-arginine methyl ester per milliliter.

†P < 0.001, compared with the control (unpaired two-way Student t test).
‡P < 0.05, compared with the control (unpaired two-way Student t test).

Cant (p < 0.001) amounts of nitrite. This nitrite production was completely inhibited with competitive inhibitors of nitric oxide synthase. Explants of bovine cartilage also displayed significant inducible nitric-oxide-synthase activity when stimulated with endotoxin (p < 0.001 for occipital cartilage and p < 0.01 for knee cartilage), human recombinant interleukin-1β (p < 0.01), or human recombinant tumor necrosis factor-α (p < 0.001) (Table I). Induction of nitrite by endotoxin, interleukin-1β, or tumor necrosis factor-α in explants of bovine occipital cartilage was dose-dependent over one to 100 nanograms of tumor necrosis factor-α per milliliter, one to ten nanograms of interleukin-1β per milliliter, and one to 100 micrograms of endotoxin per milliliter (Fig. 1). To evaluate the effects of long-term culture on nitric-oxide-synthase activity, explants of bovine occipital cartilage maintained in cell culture for three months were assayed; these demonstrated induction of nitric-oxide-synthase activity by endotoxin equivalent to that of the fresh explants (Table I).

No constitutive nitric-oxide-synthase activity was found in cultured bovine chondrocyte monolayers. However, when stimulated with one to 100 micrograms of endotoxin per milliliter, one to 100 nanograms of interleukin-1β per milliliter, or one to 100 nanograms of tumor necrosis factor-α per milliliter, cultured bovine chondrocytes released as much as 170 nanomoles of nitrite per 10^6 cells in twenty-four hours. The induction of nitrite by endotoxin, tumor necrosis factor-α, and interleukin-1β was dose-dependent (Fig. 2) and time-dependent (Fig. 3-A), with maximum stimulation at twenty-four hours. The production of nitrite could be completely inhibited by the competitive inhibitor of nitric oxide synthase, Na-nitro-L-arginine methyl ester, or by the non-competitive inhibitor, aminoguanidine (Fig. 2). The induction of nitrite was inhibited by cyclo-

![Graph showing the mean (and standard error of the mean) inducible nitric-oxide-synthase activity in passage-1 chondrocytes derived from collagenase-digested bovine occipital cartilage. Induction of nitrite (NO_2^-) occurred in a dose-response manner when the chondrocytes were exposed to endotoxin (End), human recombinant tumor necrosis factor-α (TNF-α), or human recombinant interleukin-1β (IL-1β). In each case, the formation of nitrite was inhibited by one-millimolar aminoguanidine (+), an inhibitor of nitric oxide synthase. The induction of nitric-oxide-synthase activity was also inhibited by twenty micrograms of cyclohexamide per milliliter (++), an inhibitor of protein synthesis. There were six replicates in each group. *** = p < 0.001, compared with the control (unpaired two-way Student t test).]
hexamidé (an inhibitor of protein synthesis) (Fig. 2) and by five-millimolar (but not one-millimolar) ethylene glycol bis-β-aminoethyl ether NN'T'-tetra-acetic acid (Figs. 3-B and 3-C), indicating that the induction of nitric-oxide-synthase activity was dependent on protein synthesis and required the availability of small amounts of calcium. Cell viability, as determined by mitochondrial deduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, was unaffected by any of the culture conditions.

**Nitric-Oxide-Synthase Activity in Meniscus and Ligament**

There was no constitutive nitric-oxide-synthase activity in the explants of bovine meniscus or ligament (Table I) or in the cultured cells from bovine (Table II) or human meniscus or ligament (Table III). Endotoxin, interleukin-1β, and tumor necrosis factor-α did not induce nitric-oxide-synthase activity in explants of bovine meniscus or ligament (Table I) or cell cultures derived from the outgrowth of explants of human meniscus or ligament (Table III). However, small amounts of nitrite were detected in culture medium of cells derived from bovine meniscus when stimulated with endotoxin (Table II).

**Nitric-Oxide-Synthase Activity in Synovial Tissue and Capsule**

Bovine articular synovial tissue consisted of a thin translucent membrane infiltrated with blood vessels. Fresh explants of this tissue showed inducible nitric-
NITRIC OXIDE: AN IMPORTANT ARTICULAR FREE RADICAL

TABLE III
NITRIC-OXIDE-SYNTHASE ACTIVITY IN CELLS FROM OUTGROWTH CULTURES

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Constitutive</th>
<th>Inducible with 1 μg/ml Endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meniscus</td>
<td>5 ± 2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Shoulder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synovial tissue</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Capsule</td>
<td>2 ± 2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior cruciate ligament</td>
<td>&lt;1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Posterior cruciate ligament</td>
<td>&lt;1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Medial collateral ligament</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lateral collateral ligament</td>
<td>&lt;1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Patellar ligament</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*The values are given as the mean and the standard error of the mean number of nanomoles of nitrite per 10^6 cells after twenty-four hours of incubation. There were six replicates in each group. No significant constitutive or inducible activity in any cell line was found with use of the unpaired two-way Student t test.

oxide-synthase activity (the explants released nitrite under basal conditions), and this release of nitrite could be inhibited with one milligram of Nα-nitro-L-arginine methyl ester per milliliter but could not be stimulated further with one microgram of endotoxin per milliliter, ten nanograms of interleukin-1β per milliliter, or 100 nanograms of tumor necrosis factor-α per milliliter (Table I).

There was no constitutive or inducible nitric-oxide-synthase activity in passage-1 cells that had been derived from collagenase-digested bovine synovial tissue (Table II) or in passage-2 cells derived from outgrowth cultures of the capsule or synovial tissue from a human shoulder (Table III).

Effects of Methotrexate and Dexamethasone on Inducible Nitric-Oxide-Synthase Activity in Bovine Chondrocytes

Methotrexate inhibits synthesis of biopterin (a cofactor for nitric oxide synthase)16. Corticosteroids inhibit other inducible forms of nitric oxide synthase18. Lower doses of methotrexate (0.001 to 0.1 millimole) and dexamethasone (0.001 to 0.1 millimole) did not inhibit the endotoxin-stimulated formation of nitrite in cultured bovine chondrocytes. However, higher doses of methotrexate (one millimole) and dexamethasone (one millimole) completely inhibited inducible nitric-oxide-synthase activity (Figs. 4-A and 4-B).

Discussion

Human cartilage explants in vitro synthesized high concentrations of nitrite, the stable end product of nitric oxide, when stimulated with endotoxin. This release of nitrite was inhibited by Nα-nitro-L-arginine methyl ester. Bovine cartilage and chondrocytes in vitro synthesized high concentrations of nitrite when stimulated with endotoxin or the inflammatory mediators interleukin-1β and tumor necrosis factor-α. This release of nitrite was inhibited by Nα-nitro-L-arginine methyl ester and aminoguanidine, by the protein-synthesis inhibitor cyclohexamide, by high concentrations of the calcium chelator ethylene glycol bis-β-aminoethyl ether N,N,N′,N′-tetra-acetic acid, and by high concentrations of methotrexate and dexamethasone. The ability to synthesize...
and secrete high concentrations of nitrite into medium was in contrast with the abilities of explants and cell lines derived from other articular tissues, which displayed no constitutive or inducible nitric-oxide-synthase activity. Interestingly, bovine synovial tissue had constitutive but not endotoxin or inflammatory cytokine-inducible nitric-oxide-synthase activity. This activity was not present in cultured cells derived from synovial tissue. It is possible that this constitutive nitric-oxide-synthase activity was from endothelial cells, as the tissue is vascular and as endothelial cells have a constitutive form of nitric oxide synthase. It is also interesting that passage-1 cells that had been derived from collagenase-digested bovine meniscus had very small amounts of endotoxin-inducible nitric-oxide-synthase activity. To our knowledge, this is the first demonstration of inducible nitric-oxide-synthase activity in meniscal cells, and it may be a reflection of the phenotypic similarity between meniscal fibrochondrocytes and articular chondrocytes.

The findings of a specific nitric oxide synthase inducible by endotoxin and inflammatory cytokines in explants of cartilage but not anterior cruciate, posterior cruciate, medial collateral, lateral collateral, or patellar ligaments; synovial tissue; or shoulder capsule suggest that chondrocytes represent the major source of intra-articular nitric oxide during inflammation or infection. Nitric oxide may also be produced by invading inflammatory cells. The rate of nitrite production by bovine chondrocytes was similar to that described for lapine and human articular chondrocytes and was equivalent to or greater than the activities of other inducible nitric oxide synthases. Other authors have also demonstrated that nitric-oxide-synthase activity in chondrocytes can be induced by the inflammatory mediators interleukin-1β and tumor necrosis factor-α. As far as we know, we are the first to show that explants of human and bovine cartilage can also be induced to release nitric oxide.

The inducible nitric-oxide-synthase activity of bovine articular chondrocytes was inhibited by five-millimolar, but not by one-millimolar, ethylene glycol bist-β-aminoethyl ether-N,N,N′,N′-tetra-acetic acid. These results are consistent with those reported for inducible nitric-oxide-synthase activity in lapine chondrocytes and in human chondrocytes. One-millimolar ethylene glycol bist-β-aminoethyl ether-N,N,N′,N′-tetra-acetic acid did not inhibit inducible nitric-oxide-synthase activity in human chondrocytes, but, as in bovine chondrocytes, a higher concentration may do so.

In the present study, the dose-response curves for the effects of methotrexate imply that millimolar concentrations of that substrate can inhibit inducible nitric-oxide-synthase activity in bovine chondrocytes. As methotrexate inhibits the synthesis of biotin, these results imply that biotin is important in the regulation of inducible nitric-oxide-synthase activity in chondrocytes. To our knowledge, we are the first to investigate the effect of methotrexate on such activity. The results of our study are consistent with the results for vascular smooth-muscle cells, in which methotrexate is a weak inhibitor of the synthesis of nitric oxide.

Our dose-response curves for the effects of dexamethasone on inducible nitric-oxide-synthase activity in bovine chondrocytes are consistent with those reported for rabbit chondrocytes and for human chondrocytes, in the sense that less than one-millimolar concentrations of dexamethasone were relatively ineffective in inhibiting inducible nitric-oxide-synthase activity. However, high concentrations of dexamethasone effectively inhibited such activity. These observations regarding the ability of dexamethasone and methotrexate to inhibit inducible nitric-oxide-synthase activity in chondrocytes may have clinical implications, as steroids and methotrexate are used in the treatment of inflammatory arthropathies and nitric oxide may be a major pathogenic factor in these diseases.

High concentrations of nitrite have been found in the serum and synovial fluid of patients who have osteoarthritis or rheumatoid arthritis and in animals with experimentally induced and autoimmune arthritis. Our results implicate cartilage as the major source of nitrite in these situations. Nitric oxide is toxic in high concentrations but it is even more toxic when it can combine with superoxide free radicals. Nitric oxide released from chondrocytes decreases the synthesis of proteoglycan and enhances the catabolism of proteoglycan and collagen through metalloproteinase activity. Nitric oxide also has the ability to interact directly with cyclo-oxygenase to increase the synthesis of prostaglandin E₂. These findings have additional clinical importance because inhibitors of nitric-oxide-synthase activity have inhibited the development of autoimmune and inflammatory arthritis in mice and rats.

In summary, our findings and those of others support the hypothesis that nitric-oxide-synthase activity is induced in chondrocytes during inflammation or infection of a joint. The nitric oxide released by chondrocytes may mediate a cascade of events, such as decreased synthesis of the matrix, increased degradation of the matrix, and increased synthesis of prostaglandin, that lead to the destruction of cartilage. This pathway of destruction is upstream from the points in the inflammatory cascade at which traditional non-steroidal anti-inflammatory agents are effective (such as at cyclo-oxygenase). Thus, the L-arginine-nitric oxide synthase pathway may offer novel potential sites for pharmacological inhibition of the destructive changes associated with rheumatoid arthritis and osteoarthritis.
References


