Regulation of the nitric oxide production resulting from the glucocorticoid-insensitive expression of iNOS in human osteoarthritic cartilage

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Summary

Objective: Nitric oxide (NO) produced by cartilage and synovial membranes is implicated in the pathogenesis of osteoarthritis (OA) and inhibitors of NO synthesis may have indications in the treatment or prevention of joint destruction in OA. Because the signaling mechanisms as well as the NOS isoform involved in induction of NO production in human cartilage remain in many parts unclear, the present study was designed to investigate the regulation of inducible NO synthesis in human intact OA cartilage.

Methods: Cartilage specimens were collected from OA patients undergoing knee replacement surgery and studied for iNOS expression and NO production in organ culture to allow intact chondrocyte–matrix interactions. J774 macrophages were used for comparison as a well-documented source of iNOS.

Results: OA cartilage expressed iNOS and produced NO in the absence of exogenous cytokines. Addition of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) or lipopolysaccharide (LPS) into the culture medium enhanced NO production in a dose- and time-dependent manner. Various NOS inhibitors suppressed NO production in the following order of potency: 1400W (novel selective iNOS inhibitor)=L-NIO-L-NAME+L-NMAA (an inhibitor of protein synthesis), pyrrolidine dithiocarbamate (PDTC; an NF-κB inhibitor) and genistein (an inhibitor of tyrosine protein kinases) inhibited cytokine-induced NO production, while dexamethasone, diaminohydroxyprymidine (DAHP; an inhibitor of tetrahydrobiopterin synthesis) and PD 98059 (p42/44 MAP kinase inhibitor) had no effect.

Conclusions: The results suggest that NO synthesis in human osteoarthritic cartilage derives from the glucocorticoid-insensitive expression of iNOS. Very similar mechanisms appear to regulate inducible NO synthesis in human intact OA cartilage and J774 macrophages with very similar mechanisms appear to regulate inducible NO synthesis in human intact OA cartilage and J774 macrophages with the exception that dexamethasone inhibited NO production in J774 cells but not in osteoarthritic cartilage. © 2001 OsteoArthritis Research Society International

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it is a novel isofrom of NOS or a modification of one of the already documented isoforms, either iNOS or nNOS. Inhibitors of cartilage NOS expression and NO synthesis have potential in the prevention of joint destruction, especially if osteoarthritic cartilage NOS were differently regulated than other isoforms of NOS. Selective inhibition of cartilage NOS would then have significant therapeutic implications.

Most of the studies on the regulation of NOS expression and NO production in chondrocytes have been done in cultures of isolated cells. It is known that extracellular matrix regulates chondrocyte metabolism in intact cartilage. In cell culture, the phenotype of isolated chondrocytes alters rapidly and the chondrocytes begin to proliferate. Therefore the data from studies with cultured chondrocytes are not directly applicable for intact osteoarthritic cartilage. The aim of the present study was to characterize the NOS isoform in intact osteoarthritic cartilage by studying the regulation of its expression and its sensitivity to various NOS inhibitors. The results suggest that in intact osteoarthritic cartilage IL-1-induced NO production results from the glucocorticoid-insensitive expression of iNOS, the activity of which is effectively blocked by a recently developed selective iNOS inhibitor, 1400W.

Material and methods

PATIENTS, TISSUE AND CELL CULTURES

Cartilage tissue was obtained from the leftover pieces of total knee replacement surgery. The study protocol was approved by the ethics committee of Tampere University Hospital. The donor patients, ages ranging from 48 to 88 years, were all diagnosed as having OA. Full thickness pieces of articular cartilage were removed aseptically from subchondral bone with a scalpel and cut into small pieces. These pieces of cartilage were incubated at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) (Gibco BRL, Paisley, Scotland, U.K.). J774 murine macrophages were cultured under similar conditions and harvested with trypsin-EDTA (EDTA=ethylenediaminetetraacetic acid; Gibco BRL, Paisley, Scotland, U.K.).

NITRITE DETERMINATION

The culture medium was collected after the time indicated and nitrite, a stable product of NO in aqueous solutions, was measured using the Griess reaction-prepared standards; Bio-Rad, Hercules, CA, U.S.A. The culture medium was mixed with an equal volume of 0.05 M/L Tris-HCl buffered saline pH 7.6 (TBS) and immunostained with the biotin-streptavidin alkaline phosphatase technique as previously described. The sections were incubated for 18 h with the primary antibodies that included the polyclonal sheep IgG antibody to human iNOS (1/500), the chondrocyte marker 5B5 (1/100), and the negative control normal sheep IgG. This was followed by 1 h incubation with biotinylated rabbit anti-sheep IgG antibody; sections stained with the 5B5 monoclonal antibody were incubated with biotinylated horse anti-mouse IgG antibody. Alkaline phosphatase streptavidin conjugate was added to all slides for 1 h incubation. The substrate reaction was developed using Napthol AS-BI phosphate and Fast Red TR salt. Levamisole was used at concentration of 10 mM as an inhibitor of endogenous alkaline phosphatase. To confirm the specificity of the immunostaining, the primary antibody was replaced with TBS in one section. In addition two sections from each cartilage were stained with the substrate solution with or without levamisole to exclude the possibility of non-specific labeling of endogenous alkaline phosphatase.

WESTERN BLOT ANALYSIS

Frozen cartilage specimen milled with Micro-Dismembrator and cell pellets from J774 cells were lysed in ice-cold extraction buffer (10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonylfluoride, 2 mM Na-orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotin, 1.25 mM NaF, 1 mM Na-pyrophosphate, 10 mM n-octyl-β-D-glucopyranoside; all from Sigma, St Louis, MO, U.S.A.). Following incubation in ice for 15 min, samples were centrifuged and the resulting supernatant boiled for 5 min in sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% SDS and 10 mM 2-mercaptoethanol; all from Sigma, St Louis, MO, U.S.A.) and stored at −20°C until analyzed. An aliquot of the supernatant was used to measure the protein concentration by the Coomassie blue method. Protein samples (10 μg) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose. INOS protein was identified by Western blot using rabbit polyclonal iNOS antibody (N-20) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). This antibody is raised against a peptide corresponding to an amino acid sequence mapping the amino terminus of human iNOS. It reacts with iNOS of mouse, rat and human origin and there is no cross-reactivity with neuronal or endothelial NOS. The molecular weight was estimated using molecular weight standards (Precision Prestained Standards; Bio-Rad, Hercules, CA, U.S.A.).

STATISTICAL ANALYSIS

Results are expressed as mean±standard error of the mean (S.E.M.). Statistical significance was calculated by ordinary or repeated measures analysis of variance followed by Bonferroni multiple comparisons test or by Friedman non-parametric repeated measures test followed by Dunn’s multiple comparisons test. Differences were considered significant when P<0.05.

REAGENTS

IL-1β was purchased from Genzyme, Cambridge, MA, U.S.A. and TNFα from Immunogenex Corp. (Los Angeles, CA, U.S.A.). Affinity purified polyclonal sheep IgG antibody
to human iNOS was a gift from Dr I. Charles. The monoclonal antibody 5B5 to the β-subunit of prolyl-4-hydroxylase, a marker of fibroblasts and chondrocytes, was purchased from Dako. Bacterial lipopolysaccharide (LPS), cycloheximide, NG-nitro-L-arginine methyl ester (L-NAME) and genistein were from Sigma, St Louis, MO, U.S.A. N-monomethyl-L-arginine (L-NMMA; Clinalfa, Laufelfingen, Switzerland), L-N-iminoethyl-ornithine (L-NIO) and 1400W (Glaxo Wellcome, Stevenage, U.K.), dexamethasone (Orion Corp., Espoo, Finland), PD 98059 (Calbiochem-Novabiochem Corporation, La Jolla, Canada), pyrrolidine dithiocarbamate (PDTC) and diaminohydroxypyrimidine (DAHP; Tocris, Langford, Bristol, U.K.) were obtained as indicated.

Results

SPONTANEOUS NITRIC OXIDE PRODUCTION BY OA CARTILAGE

OA cartilage produced low concentrations of nitrite in organ culture in the absence of exogenous cytokines [Fig. 1(a)]. Nitrite accumulation was linear up to 72 h follow-up and it was partly inhibitable with a nitric oxide synthase (NOS) inhibitor L-NIO at 1 mM concentrations suggesting that NO was produced by the unstimulated cartilage.

Fig. 1. (a) Nitrite production by osteoarthritic cartilage in the absence of exogenous cytokines (−●−) and the inhibitory effect of NOS inhibitor L-NIO (1 mM, −■−). Mean±S.E.M., 7 patients, each with 6 replicates. Statistical significance was calculated by Friedman non-parametric repeated measures test followed by Dunn’s multiple comparisons test. *** indicates P<0.001 as compared to samples incubated in the presence of L-NIO (N=7 was used in statistical analysis). (b) Immunohistochemical section showing positive staining for iNOS in osteoarthritic cartilage.

Human iNOS expression was also assessed by Western blotting (see Fig. 4), and by immunohistochemistry in sections of cartilage from seven OA patients undergoing joint replacement operation. All sections demonstrated positive labeling of the chondrocytes for β subunit of prolyl-4-hydroxylase with the Mab 5B5. The purified sheep antibody to iNOS used in this study showed high intensity of immunoreactivity with more than 70% of chondrocytes in the cartilage sections from the seven patients [Fig. 1(b)]. The majority of the single chondrocytes within the superficial outer surface of the cartilage were positive. In the deeper layers the staining was less uniform, and was seen in chondrocytes within the clusters of the adjacent lacunae or doublets (2–4 cells in one lacuna). The staining was almost restricted to perinuclear cytoplasm. No positive staining was observed outside the lacunae in any of the cases.

CYTOKINE STIMULATED NITRIC OXIDE PRODUCTION BY OA CARTILAGE

Proinflammatory cytokines IL-1β (0.04–4 ng/ml) and TNFα (0.4–40 ng/ml) stimulated nitrite production in OA cartilage in a concentration dependent manner [Fig. 2(a)].
A non-selective NOS inhibitor, L-NIO (1 mM), prevented IL-1β- and TNFα-induced nitrite accumulation indicating that it was due to NO production. Bacterial endotoxin (LPS) was a less potent stimulus than IL-1β and TNFα and only a concentration of 1000 ng/ml increased NO production significantly [Fig. 2(c)]. Figure 3 shows that NO production in response to each of these three stimuli was linear up to 72 h follow-up. Western blot analysis was carried out with an antibody against human iNOS, which reacts with mouse, rat and human iNOS but not with neuronal or endothelial NOS. In cartilage pieces treated for 48 h with IL-1β (4 ng/ml), TNFα (40 ng/ml) and LPS (1000 ng/ml) a clear immunoreactive band was seen, and it was of similar MW as iNOS in LPS-treated J774 murine macrophages. OA cartilage incubated in the absence of exogenous cytokines also expressed iNOS but at lower level than cytokine-treated cartilage (Fig. 4).

EFFECTS OF VARIOUS NOS INHIBITORS ON IL-1β-INDUCED NO PRODUCTION IN OA CARTILAGE

IL-1β stimulated nitrite production in the OA-affected cartilage was inhibitable with various NOS inhibitors in a dose-dependent manner showing the following order of inhibition: 1400W (selective iNOS inhibitor)=L-NIO>L-NMMA>L-NAME which was quite inefficient [Fig. 5(a)]. For comparison, these four NOS inhibitors were also tested on LPS-induced NO production in J774 macrophages and a similar order of potency was seen [Fig. 5(b)].

MECHANISMS INVOLVED IN THE IL-1β-INDUCED NO PRODUCTION IN OA CARTILAGE

The mechanisms of the IL-1β-stimulated NO production were studied by pharmacological means. Cycloheximide (an inhibitor of protein synthesis; 10 μg/ml), PDTC (an inhibitor of the transcription factor NF-κB; 100 μM) and genistein (an inhibitor of tyrosine protein kinase; 100 μM) significantly inhibited the IL-1β-induced nitrite production in osteoarthritic cartilage, while dexamethasone (10 μM), PD 98059 (p42/44 MAP kinase inhibitor; 10 μM) and DAHP (an inhibitor of tetrahydrobiopterin synthesis; 1 mM) had no effect [Fig. 6(a)]. For comparison, the effects of these compounds were also tested on LPS-induced NO production in J774 macrophages: NO production was respectively inhibitable with cycloheximide, PDTC and genistein. P42/44 MAP kinase inhibitor PD 98059 and tetrahydrobiopterin inhibitor DAHP caused a slight but statistically significant inhibition on LPS-induced NO production in J774 macrophages; a similar trend not reaching statistical significance was seen also in cartilage specimen [Fig. 6(b)]. In the case of dexamethasone there was a clear difference in its action on NO synthesis in osteoarthritic cartilage and J774 macrophages. The former was insensitive for the action of dexamethasone whereas LPS-induced NO production in J774 cells was suppressed by 40% in the presence of 10 μM dexamethasone (Fig. 6).

Discussion

Chondrocytes were the second cell type in which human iNOS was cloned and characterized soon after it was found in hepatocytes. Although the cDNA sequence of human iNOS is quite similar (88%) to the sequence of the
murine macrophage iNOS, there are major differences in the signaling mechanisms resulting in induction of NOS between human and mouse cells. Unlike murine iNOS, human iNOS in inflammatory and most other cells is not inducible with LPS or a single cytokine but requires a combination of various cytokines or other more complex treatments\textsuperscript{20,26,27}. Human chondrocytes are among few human cell types in which NO production is induced in response to LPS alone or to a single cytokine, of which IL-1 and TNF\textsubscript{a} were used in the present study. In addition to the 131 kDa human iNOS which was first described and cloned in IL-1-treated human chondrocytes\textsuperscript{16}, there are also reports on another NO synthase found in osteoarthritic cartilage (OA-NOS) which resembles neuronal NOS rather than iNOS in its molecular weight and antibody binding\textsuperscript{22}. In the present study we compared the IL-1-induced NO production in human articular cartilage to LPS-induced NO synthesis by iNOS-pathway in murine J774 macrophages. NO synthesis in these two cell types was quite similarly regulated in terms of sensitivity to various NOS inhibitors and basic signaling mechanisms involved in the transcriptional activation of iNOS gene with the exception of dexamethasone treatment which suppressed NO production in J774 macrophages but not in human cartilage.

The present data showed that pieces of osteoarthritic cartilage maintained in tissue culture produced nitrite also in the absence of exogenous cytokines or LPS. Nitrite accumulation was partly inhibited by high (1 mM) concentrations of a NOS inhibitor L-NIO indicating that NO was produced by unstimulated cartilage explants. However, it is not clear what is the origin of the L-NIO-insensitive nitrite that accumulated into the culture. Because L-NIO is a non-selective NOS inhibitor and it was used in a high concentration (1 mM), it is unlikely that the L-NIO-insensitive nitrite formation was due to NO production. To exclude non-specific reactivity of interfering compounds with the Griess reagent as a reason for the L-NIO-insensitive nitrite accumulation, nitrite measurements were confirmed also by ozone-chemiluminescence method. These data confirm that the part of nitrite production that was not inhibited by a NOS inhibitor is due to nitrite formation by other biochemical pathways or may result from the poor penetration of the inhibitors into cartilage. Subtotal inhibition of nitrite accumulation by various NOS inhibitors used up to 10 mM concentrations in chondrocyte cultures\textsuperscript{28} supports the former hypothesis. Accordingly L-NIO was effective in the present experiments in which cartilage was treated with TNF or LPS. In the further studies iNOS expression in osteoarthritic cartilage was confirmed by immunostaining of cartilage samples snap-frozen immediately after surgery. These data, together with the findings of Sakurai \textit{et al.}\textsuperscript{19} and Amin \textit{et al.}\textsuperscript{22}, give direct evidence that NOS is expressed in osteoarthritic cartilage \textit{in vivo} and suggest that NO has a role in the pathogenesis of OA.

IL-1-induced NO production in cartilage as well as in J774 cells was sensitive to cycloheximide suggesting that it was dependent on \textit{de novo} NOS protein synthesis.
Enhanced expression of iNOS protein in cartilage pieces exposed to IL-1, TNF-α or LPS was studied by Western blot analysis using an antibody that reacts with iNOS but not with neuronal or endothelial NOS. IL-1β, TNF-α and LPS induced expression of protein that stained with this antibody and was of similar size as the iNOS expressed in LPS-treated J774 macrophages. These data are consistent with the idea that the NOS isoform in osteoarthritic cartilage is iNOS. PDTC was used to inhibit the activation of nuclear factor-κB (NF-κB) which is known to be critical for the induction of iNOS gene transcription. NO production in IL-1-treated cartilage and LPS-treated J774 cells was equally inhibited by PDTC suggesting that activation of NF-κB is involved in both of these activation mechanisms. In addition, IL-1-induced NO production in osteoarthritic cartilage and LPS-induced NO synthesis in J774 macrophages were similarly regulated by genistein (tyrosine kinase inhibitor), PD 98059 (p42/44 MAP kinase inhibitor) and DAHP (BH4 synthesis inhibitor). In the further experiments we studied the sensitivity of NO production in IL-1-treated human cartilage and LPS-treated murine J774 macrophages to various NOS inhibitors. Nitrite production was measured by Griess reaction and expressed as pmol nitrite/mg tissue or µmol/l.

Fig. 5. The inhibition of nitrite production by various NOS inhibitors. (a) The effect on osteoarthritic cartilage stimulated with IL-1β (4 ng/ml) for 42 h and on (b) J774 macrophages stimulated with LPS 100 ng/ml for 24 h. NOS inhibitors were added into the culture medium at the beginning of the incubation. Nitrite was measured by Griess reaction and expressed as pmol nitrite/mg tissue or µmol/l. The culture medium contained 100 µM L-Arginine. The horizontal line shows nitrite concentration produced by osteoarthritic cartilage in the absence of exogenous IL-1β. Mean±S.E.M., 5 patients, each with 4 replicates. Statistical significance was calculated with repeated measures (a) or ordinary (b) ANOVA. *=P<0.05, **=P<0.01, ***=P<0.001 as compared to control samples incubated in the absence of NOS inhibitors [in (a) N=5 was used in the statistical analysis; in (b) N=6]. IL-1β or LPS alone, □ IL-1β or LPS plus NOS inhibitor: 10 µM, □; 100 µM, □; 1000 µM, □.
by both chondrocytes and macrophages was inhibitable in a concentration dependent manner by 1400W, L-NIO, L-NMMA and L-NAME which showed similar order of potency in both cell types. An iNOS selective inhibitor 1400W was the most potent inhibitor while L-NAMe that shows some selectivity towards the neuronal and endothelial type of NOS (vs iNOS) was quite inefficient, reaching a 31% and 51% inhibition at 1 mM concentrations in articular cartilage and J774 cells, respectively. The dose-response curves of L-NIO and L-NMMA were in between those of 1400W and L-NAME. N-(3-(aminomethyl)benzyl)acetamidine (1400W) is a recently characterized tightly binding (irreversible or extremely slowly reversible) very selective inhibitor of iNOS vs both eNOS and nNOS. This is the first report to show that 1400W is a potent NOS inhibitor also in IL-1-treated human articular cartilage indicating that it penetrates well in cartilage tissue and may be a useful tool for further studies on cartilage NO production. Thus our results support the idea that iNOS is the predominant isotype of nitric oxide synthase involved in IL-1-induced NO production in human osteoarthritic cartilage.

So far, iNOS is the only NOS enzyme that has been cloned in human chondrocytes and immunoreactivity in Western blot gels is close to rat brain nNOS and different from human hepatocyte and murine macrophage iNOS. At the moment it is not clear whether their OA-NOS is identical to nNOS, a novel NOS iso-enzyme or a modification of iNOS. Our data suggest that IL-1 treated osteoarthritic cartilage produce NO by iNOS pathway. We also found iNOS expression in untreated osteoarthritic cartilage. These data made us to propose that OA-NOS is merely a modification of iNOS. In addition, difference in experimental procedures and antibodies used may explain some differences between our results and those reported by Amin et al.

Dexamethasone, although used at a high concentration (10 μM) failed to inhibit NO production in IL-1-treated cartilage whereas LPS-induced NO synthesis was inhibited by about 40%. This is consistent with the earlier data that chondrocyte NO production is resistant to antiinflammatory steroids. In other cell types glucocorticoids seem to have variable effects. The detailed mechanisms of the inhibitory action by glucocorticoids on NO production seen in some cell types remain unknown but different post-transcriptional effects have been reported. Therefore the differential action of dexamethasone on NO production in IL-1-treated cartilage and LPS-treated macrophages does not necessarily mean that the signalling mechanisms leading to induction of NOS between these cell types are different.

The present data show that osteoarthritic cartilage produces NO, and its synthesis is further enhanced by proinflammatory cytokines IL-1 and TNFα and by bacterial endotoxin. A novel selective iNOS inhibitor 1400W was a potent inhibitor of NO synthesis in human articular cartilage, whereas dexamethasone had no effect. IL-1-induced NO production was dependent on protein synthesis, activation of transcription factor NF-κB and tyrosine kinases while inhibitors of p42/44 MAP kinase and tetrahydrobiopterin synthesis were ineffective. In addition, Western blot analysis showed iNOS in human articular cartilage, which was of similar MW (about 131 kDa) as iNOS in LPS-treated J774 murine macrophages. The results suggest that IL-1-induced NO production in osteoarthritic cartilage results from NF-κB sensitive and dexamethasone insensitive expression of iNOS. These data are implicated in the development of novel treatments against OA and RA because there is increasing amount of evidence on a novel OA-NOS that based on its MW and evidence on a novel OA-NOS that based on its MW and...
References
