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Histone deacetylase inhibitors suppress interleukin-1β-induced nitric oxide and prostaglandin E_2 production in human chondrocytes

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Summary

Objective: Overproduction of nitric oxide (NO) and prostaglandin E_2 (PGE_2) plays an important role in the pathogenesis of osteoarthritis (OA). In the present study, we determined the effect of trichostatin A (TSA) and butyric acid (BA), two histone deacetylase (HDAC) inhibitors, on NO and PGE_2 synthesis, inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 expression, and nuclear factor (NF)-κB DNA-binding activity, in interleukin-1β (IL-1)-stimulated human OA chondrocytes, and on IL-1-induced proteoglycan degradation in cartilage explants.

Methods: Chondrocytes were stimulated with IL-1 in the absence or presence of increasing concentrations of TSA or BA. The production of NO and PGE_2 was evaluated using Griess reagent and an enzyme immunoassay, respectively. The expression of iNOS and COX-2 proteins and mRNAs was evaluated using Western blotting and real-time reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. Proteoglycan degradation was measured with dimethymethylene blue assay. Electrophoretic mobility shift assay (EMSA) was utilized to analyze the DNA-binding activity of NF-κB.

Results: HDAC inhibition with TSA or BA resulted in a dose-dependent inhibition of IL-1-induced NO and PGE_2 production. IL-17- and tumor necrosis factor-α (TNF-α)-induced NO and PGE_2 production was also inhibited by TSA and BA. This inhibition correlated with the suppression of iNOS and COX-2 protein and mRNA expression. TSA and BA also prevented IL-1-induced proteoglycan release from cartilage explants. Finally, we demonstrate that the DNA-binding activity of NF-κB, was induced by IL-1, but was not affected by treatment with HDAC inhibitors.

Conclusions: These data indicate that HDAC inhibitors suppressed IL-1-induced NO and PGE_2 synthesis, iNOS and COX-2 expression, as well as proteoglycan degradation. The suppressive effect of HDAC inhibitors is not due to impaired DNA-binding activity of NF-κB. These findings also suggest that HDAC inhibitors may be of potential therapeutic value in the treatment of OA.

Key words: Chondrocytes, Nitric oxide, Inducible nitric oxide synthase, Prostaglandin E_2, Cyclooxygenase-2, Histone deacetylases, Nuclear factor-κB.

Abbreviations: BA butyric acid, COX-2 cyclooxygenase-2, HDAC histone deacetylase, IL interleukin, iNOS inducible nitric oxide synthase, NF-κB nuclear factor-κB, NO nitric oxide, OA osteoarthritis, PGE_2 prostaglandin E2, TNF-α tumor necrosis factor-α, TSA trichostatin A.

Introduction

Osteoarthritis (OA) is the most common joint disorder and a leading cause of disability among the elderly population. It is characterized by progressive degenerative structural changes in articular cartilage, leading to loss of joint function. It is also characterized by excessive production of several inflammatory mediators[1–3]. Among these mediators, the pro-inflammatory cytokine interleukin-1β (IL-1) plays a pivotal role in the pathophysiology of OA. It induces a cascade of inflammatory and catabolic events in chondrocytes including the synthesis of prostaglandin E_2 (PGE_2) and nitric oxide (NO). IL-1 also alters chondrocyte anabolism by suppressing the synthesis of proteoglycan and collagen and by enhancing the production of matrix metalloproteinases (MMPs)[1–3].

NO is synthesized from L-arginine by a family of NO synthases (NOSs) of which three isoforms have been identified. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while the inducible NOS (iNOS) is expressed following stimulation with a variety of inflammatory agents such as endotoxins or cytokines[4]. NO promotes inflammation by enhancing the production of inflammatory cytokines[5] and PGE_2[6] and by reducing

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the synthesis of endogenous IL-1 receptor antagonist (IL-1 Ra)[7]. NO is also considered a potent catabolic agent in OA since it inhibits collagen and proteoglycan synthesis[8,9], stimulates the production and activation of MMPs[10] and induces chondrocyte apoptosis[11]. Accordingly, the *in vivo* selective inhibition of iNOS in an experimental model of OA reduces the joint structural changes and the expression of several inflammatory and catabolic factors, including IL-1 and MMP-1[12].

The biosynthesis of PGE_2 from arachidonic acid (AA) involves multiple enzymes including, cyclooxygenases (COXs). Two isoforms of COX have been identified: COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced by various stimuli such as endotoxins, growth factors and pro-inflammatory cytokines[13]. PGE_2 is the most abundant prostanoid in arthritic joint and one of the major catabolic mediators involved in cartilage resorption. PGE_2 elicits cartilage resorption by enhancing the activation and production of MMPs and the degradation of cartilage matrix components[14,15] and by promoting chondrocyte apoptosis[16]. In addition PGE_2 mediates pain responses and potentiates the effects of other inflammatory mediators[13].

Acetylation and deacetylation of nucleosomal histones play an important role in the regulation of gene expression[17,18]. The histone acetylation status is controlled by the opposing actions of two classes of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs). Acetylation of histones loosens nucleosomal structures, thereby promoting gene transcription. In contrast, deacetylation of histones stabilizes nucleosomal structures and represses gene transcription[17,18]. However, emerging evidence indicates that gene regulation by acetylation/deacetylation is more dynamic and complex, and that HATs can act as repressors and HDAC as activators of transcription. Indeed, global analysis of gene expression has shown that inhibition of HDAC activity results both in induction and repression of gene expression[19–24].

In recent years, significant interest has emerged in the inhibition of HDAC activity as a possible anti-cancer treatment. HDAC inhibitors induce growth arrest, differentiation and apoptosis of cancer cells *in vitro* and reduce the growth of experimental tumors *in vivo*[25,26]. Presently, several HDAC inhibitors are in clinical trials for the treatment of solid and hematological tumors[27,28]. In addition to their anti-cancer effects, recent studies have demonstrated that HDAC inhibitors modulate inflammatory responses. For instance, HDAC inhibitors reduce the production of IL-1, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells[29,30]. Likewise, HDAC inhibitors prevent LPS-induced production of TNF-α, IL-6 and reactive oxygen species in neuroglia cultures, and primary microglia[31–33]. HDAC inhibitors have also been reported to suppress IL-12 production in dendritic cells and macrophages[34]. However, it is currently unknown whether HDAC inhibitors regulate inflammatory responses in articular chondrocytes.

Since excessive production of the inflammatory mediators NO and PGE_2 plays an important role in the pathogenesis of OA, we assessed the effect of two HDAC inhibitors, trichostatin A (TSA) and butyric acid (BA), on the production of NO and PGE_2 in primary cultured human chondrocytes stimulated with IL-1. We additionally analyzed the expression of iNOS and COX-2 as well as the binding activity of transcription factor nuclear factor-κB (NF-κB).

Materials and methods

REAGENTS

Recombinant human (rh) IL-1β was obtained from Genzyme (Cambridge, MA, USA), rhTNF-α and rhIL-17 were from R&D Systems (Minneapolis, MN, USA). TSA and BA were from Sigma–Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, fetal calf serum (FCS), and TRIzol® reagent were from Invitrogen (Burlington, ON, Canada). All other chemicals were purchased from either Sigma–Aldrich Canada or Bio-Rad (Mississauga, ON, Canada).

SPECIMEN SELECTION AND CHONDROCYTE CULTURE

Human normal cartilage (from femoral condyles) was obtained at necropsy, within 12 h of death, from donors with no history of arthritic disease ($n = 7$, mean ± SD age: 54 ± 16 years). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically. Only those with no alterations were further processed. Human OA cartilage samples from femoral condyles and tibial plateaus were obtained from OA patients undergoing total knee replacement ($n = 47$, mean ± SD age: 66 ± 12 years). All OA patients were diagnosed according to the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA[35]. At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of non-steroidal anti-inflammatory drugs (NSAIDs) or selective COX-2 inhibitors. Patients who had received intra-articular injections of steroids were excluded. The Clinical Research Ethics Committee of Notre-Dame Hospital approved the study protocol and the use of human articular tissues.

Chondrocytes were released from cartilage by sequential enzymatic digestion as previously described[36]. In brief, this consisted of 2 mg/ml pronase for 1 h followed by 1 mg/ml collagenase (type IV; Sigma–Aldrich) for 6 h at 37°C in DMEM and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The digested tissue was briefly centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks and cultured in DMEM supplemented with 10% heat-inactivated FCS. At confluence, the chondrocytes were detached, seeded at high density, and allowed to grow in DMEM, supplemented as above. The culture medium was changed every second day, and 24 h before the experiment the cells were incubated in fresh medium containing 0.5% FCS. Only first passaged chondrocytes were used.

NO AND PGE_2 DETERMINATIONS

The nitrite levels, used as an indicator of NO production, were determined using the Griess assay as previously described[36]. The levels of PGE_2 were determined using a PGE_2 enzyme immunoassay from Cayman Chemical (Ann Arbor, MI, USA). The detection limit and sensitivity was 9 pg/ml. All assays were performed in duplicate.

WESTERN BLOT ANALYSIS

Chondrocytes were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethansulfonylfluorid (PMSF), 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were sonicated on ice and centrifuged at 12,000 rpm for 15 min. The protein concentration of the supernatant was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Twenty microgram of total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with a Tris buffer [Tris-buffered saline (TBS) pH 7.5, with 0.1% Tween 20]. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce), washed again, incubated with SuperSignal Ultra Chemiluminescent reagent (Pierce), and exposed to Kodak X-Omat film (Eastman Kodak Ltd, Rochester, NY, USA).

RNA EXTRACTION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA from stimulated chondrocytes was isolated using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA), dissolved in diethylpyrocarbonate (DEPC)-treated-H_2O and stored at −80°C until use. One microgram of total RNA was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, Burlington, ON, Canada) as