Osteoarthritis and Cartilage (2008) **16,** 1267–1274

doi:10.1016/j.joca.2008.03.009

Osteoarthritis and Cartilage

International Cartilage Repair Society

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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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Received 23 October 2007; revision accepted 9 March 2008.

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

detailed in the manufacturer's guidelines. One fiftieth of the reverse transcriptase reaction was analyzed by real-time PCR as described below. The following primers were used: iNOS, sense 5′-ACATTGATGAGAAGC TGTCCCAC-3′ and antisense 5′-CAAAGGCTGTGAGTCCTGCAC-3′; COX-2, sense 5′-TGTGTTGACATCCAGATCAC-3′ and antisense 5′-ACAT CATGTTTGAGCCCTGG-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5′-CAGAACATCATCCCTGCCTCT-3′ and antisense 5′-GCTTGACAAAGTGGTCGTTGAG-3′.

REAL-TIME PCR

Real-time PCR analysis was performed in a total volume of 50 μl containing template DNA, 200 nM of sense and antisense primers, 25 μl of SYBR® Green master mix (QIAGEN, Mississauga, ON, Canada) and uracil-*N*-glycosylase (UNG, 0.5 Unit, Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 min (UNG reaction), and at 95°C for 10 min (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real-time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA) allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with one peak, indicating the specificity of the amplification. A threshold cycle (C_T value) was obtained from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative mRNA expression in chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔC_T value was first calculated by subtracting the C_T value for the housekeeping gene GAPDH from the C_T value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the control (unstimulated cells) from the ΔC_T value of each treatment. Fold changes compared with the control were then determined by raising two to the $-\Delta\Delta C_T$ power. Each PCR reaction generated only the expected specific amplicon as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on two separate occasions for each independent experiment.

PROTEOGLYCAN RELEASE

Cartilage proteoglycan degradation was assessed by measuring sulfated glycosaminoglycan (GAG) released into culture media using dimethyl methylene blue (DMMB) with chondroitin sulfate as a standard[37]. Results are expressed as μg of GAG released/mg cartilage.

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts were prepared as previously described[38]. Briefly, chondrocytes were washed in ice-cold phosphate buffered saline (PBS) and gently scraped in ice-cold hypotonic buffer containing 10 mM hydroxyethylpiperazine ethane sulfonic acid (HEPES)–KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiotheritol (DTT), 1 mM PMSF, 1 mM Na_3VO_4 and 10 μg/ml of aprotinin, leupeptin, and pepstatin. The cells were allowed to swell on ice and the nuclei were recovered by brief centrifugation. The pellets were resuspended in high salt buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 25% glycerol, 0.5 mM PMSF, 1 mM Na_3VO_4 and 10 μg/ml of aprotinin, leupeptin, and pepstatin, followed by incubation on ice for 20 min. The nuclear extracts were recovered by centrifugation and protein concentration was determined using the Bradford method (Bio-Rad). A synthetic double-stranded oligonucleotide containing the κB consensus sequence 5′-AGTTGAGGGGACTTTCCCAGGC-3′ was end-labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P] adenosine triphosphate (ATP). The mutant competitor oligonucleotide had the following sequence with a 1 bp substitution (underlined): 5′-AGTTGAGGC-GACTTTCCCAGGC-3′. The binding buffer consisted of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol and 2.5 μg poly (dI–dC). Binding reactions were conducted with 5 μg nuclear extract and 100,000 cpm ^{32}[P]-labeled oligonucleotide probe at 22°C for 20 min in a final volume of 10 μl. In supershift assays, the antibody to p65 (1 μg/reaction) was incubated with the reaction mixture for 1 h at 4°C before the addition of ^{32}[P]-labeled oligonucleotide. In cold competition assays, 50-fold molar excess of cold wild-type or mutant oligonucleotide was used. Binding complexes were resolved on non-denaturating 6% polyacrylamide gel electrophoresis in Tris–borate buffer system, after which the gels were fixed, dried, and subjected to autoradiography.

STATISTICAL ANALYSIS

Data are expressed as the mean ± S.E.M. Statistical significance was assessed by the two-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

TSA AND BA ATTENUATE IL-1-INDUCED NO AND PGE_2 PRODUCTION IN HUMAN CHONDROCYTES

Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of two HDAC inhibitors, TSA and BA, and the production of NO was evaluated using Griess reagent. As shown in Fig. 1(A), treatment with either TSA or BA suppressed IL-1-induced NO production in a dose-dependent manner. Similarly, the production of PGE_2 was dose-dependently suppressed in the presence of each HDAC inhibitor [Fig. 1(B)]. In another set of experiments, we found that TSA and BA also dose-dependently inhibited IL-1-induced NO and PGE_2 production in normal chondrocytes, (n = 3, data not shown). The observed inhibition was not a result of reduced cell viability as confirmed by the methyl thiazolyl tetrazolium (MTT) assay (data not shown).

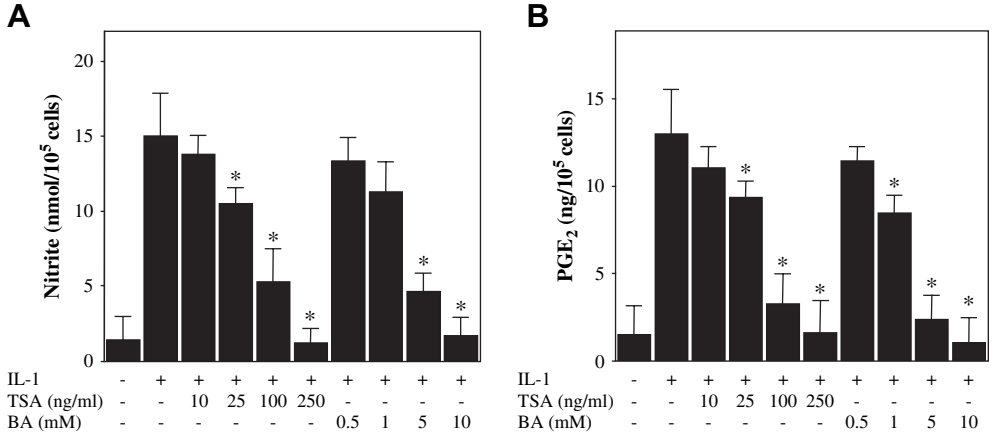

Fig. 1. HDAC inhibitors TSA and BA prevent IL-1-induced NO and PGE_2 release from chondrocytes. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA or BA for 24 h. Conditioned media were collected and analyzed for NO (A) and PGE_2 (B) release. Results are expressed as percentage of control (i.e., cells treated with IL-1 alone) and are the mean ± S.E.M. from four independent experiments. $^*P < 0.05$ compared with cells treated with IL-1 alone.

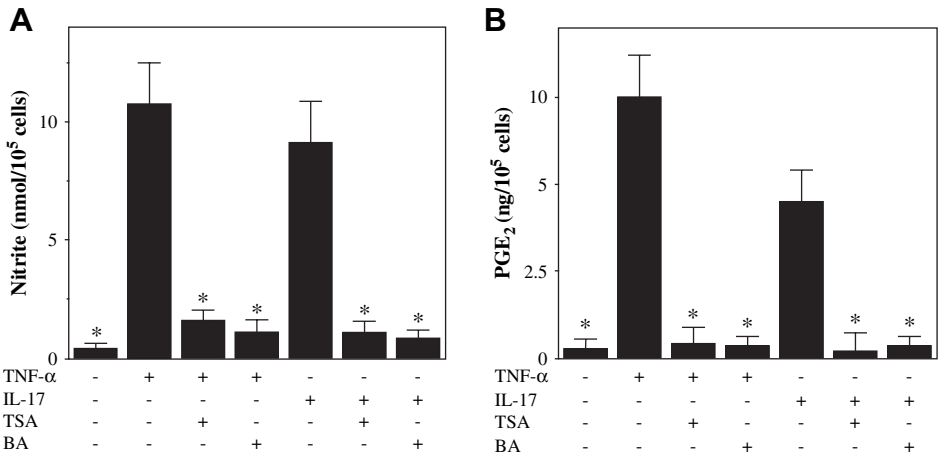

Fig. 2. TSA and BA suppress TNF-α and IL-17-induced iNOS and COX-2 protein expression. Chondrocytes were treated with TNF-α (1 ng/ml) or IL-17 (100 ng/ml) in the absence or presence of TSA (250 ng/ml) or BA (10 mM) for 24 h. Culture media were collected and analyzed for the production of NO (A) and PGE_2 (B). Results are expressed as mean ± S.E.M. of three independent experiments. *$P < 0.05$ compared with cells treated with TNF-α or IL-17 alone.

TSA AND BA INHIBIT TNF-α AND IL-17-INDUCED NO AND PGE_2 PRODUCTION

The pro-inflammatory cytokines TNF-α and IL-17 also contribute to the pathogenesis of OA and are potent inducers of NO and PGE_2 production[1–3]. Therefore, we examined whether HDAC inhibition could also attenuate TNF-α and IL-17-induced NO and PGE_2 production. As shown in Fig. 2, stimulation of chondrocytes with TNF-α or IL-17 dramatically increased the production of NO and PGE_2. Interestingly, the induction of NO and PGE_2 production by TNF-α or IL-17 was almost completely abolished after treatment with TSA or BA. These data suggest that the suppressive effect of HDAC inhibitors was not specific to IL-1, and that HDAC inhibitors might target common pathways implicated in NO and PGE_2 production.

TSA AND BA DECREASE IL-1-INDUCED iNOS AND COX-2 EXPRESSION IN CHONDROCYTES

To determine whether the inhibition of IL-1-induced NO and PGE_2 production is due to reduced iNOS, and COX-2 protein expression, the effects of HDAC inhibitors on the expression of both proteins were analyzed by Western blotting. Under basal conditions, iNOS and COX-2 proteins were undetectable and treatment with IL-1 resulted in a strong induction of both protein expressions (Fig. 3). Consistent with their effects on NO and PGE_2 production, HDAC inhibitors prevented the induction of iNOS and COX-2 protein expression by IL-1, in a concentration-dependent manner (Fig. 3). The levels of β-actin and COX-1 were not influenced by IL-1 alone or in combination with each HDAC inhibitors (Fig. 3). IL-1-induced iNOS and COX-2 protein expression was also inhibited by TSA and BA in normal chondrocytes ($n = 3$, data not shown). As expected, the induction of iNOS and COX-2 proteins by TNF-α or IL-17 was also suppressed by each HDAC inhibitor (Fig. 4).

Next, we used real-time PCR to determine whether HDAC inhibitors modulate iNOS and COX-2 mRNAs' induction. The relative expression level of each gene mRNA was plotted as fold changes over untreated control cells. GAPDH gene expression was used for normalization. As expected, IL-1 induced a marked increase of both iNOS and COX-2 mRNA levels (Fig. 5). Treatment with either TSA or BA dose-dependently suppressed the induction of iNOS and COX-2 mRNA expression (Fig. 5), suggesting that HDAC inhibitors exert their effects at the transcriptional

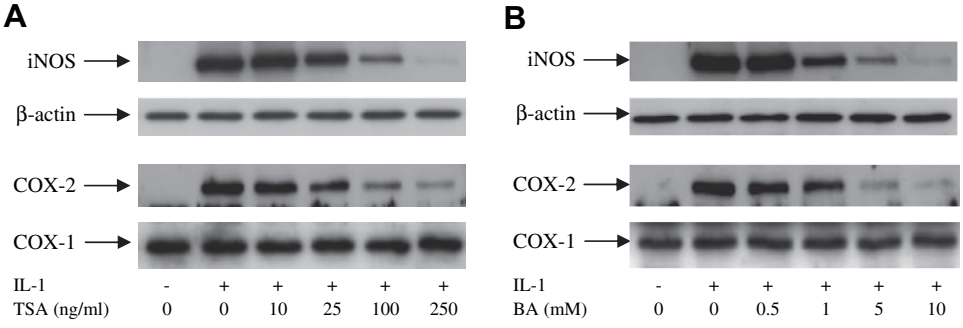

Fig. 3. TSA and BA decrease IL-1-induced iNOS and COX-2 protein expression. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA (A) or BA (B) for 24 h. Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. In the lower panels the blots were stripped and re-probed with specific anti-β-actin or anti-COX-1 antibodies. The blots are representative of similar results obtained from four independent experiments.

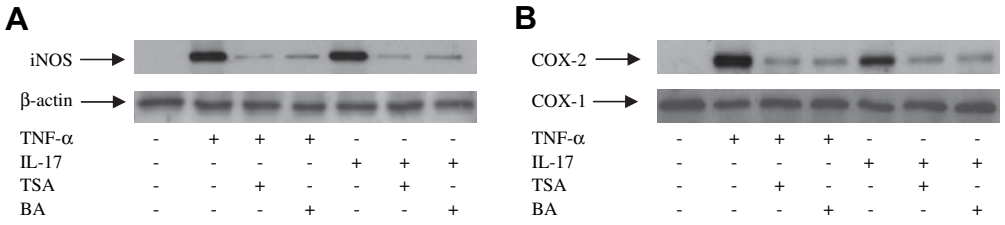

Fig. 4. TSA and BA suppress TNF-α and IL-17-induced iNOS and COX-2 protein expression. Chondrocytes were treated with TNF-α (1 ng/ml) or IL-17 (100 ng/ml) in the absence or presence of TSA (250 ng/ml) or BA (10 mM) for 24 h. Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. In the lower panels, the blots were stripped and re-probed with specific anti-β-actin or anti-COX-1 antibodies. The blots are representative of similar results obtained from three independent experiments.

level. Similar results were observed with normal chondrocytes ($n = 3$, data not shown).

TSA AND BA PREVENT IL-1-INDUCED PROTEOGLYCAN DEGRADATION IN CARTILAGE EXPLANTS

To investigate the effect of HDAC inhibitors on IL-1-induced proteoglycan degradation, cartilage explants were incubated in DMEM with 10% FBS for 48 h and then transferred to medium containing 0.5% FBS and re-incubated for an additional 48 h. Thereafter, the explants were stimulated with IL-1 in the absence or presence of increasing concentrations of TSA or BA for 72 h, and GAG release into the supernatants was determined[37]. As shown in Fig. 6, IL-1-induced GAG release was inhibited in a dose-dependent manner by either TSA or BA.

TSA AND BA DO NOT IMPAIR NF-κB-BINDING TO THE iNOS AND COX-2 PROMOTERS

The transcription factor NF-κB, mainly composed of p50 and p65 dimers, plays a pivotal role in mediating the effects of IL-1 in chondrocytes[39,40]. Therefore, we performed EMSA to determine whether HDAC inhibitors modulate IL-1 effects by interfering with the DNA-binding activity of NF-κB. Nuclear extracts from chondrocytes treated with IL-1 alone, or in combination with increasing concentrations of HDAC inhibitors for 1 h, were used for these assays. As shown in Fig. 7, IL-1 treatment induced a prominent increase in the DNA-binding activity of NF-κB. Interestingly, treatment with each HDAC inhibitor did not decrease the DNA-binding activity of NF-κB at any concentration point. The specificity of DNA-binding was confirmed using unlabeled wild-type and mutant oligonucleotides. The specificity was further evidenced using supershift assays and a specific anti-p65 antibody. Together these data suggest that HDAC inhibitors modulate IL-1 effects in chondrocytes without interfering with the DNA-binding activity of NF-κB.

Discussion

In the present study we demonstrated that inhibition of HDACs by two structurally unrelated HDAC inhibitors, TSA and BA, results in a dose-dependent suppression of IL-1-induced NO and PGE_2 production. The inhibition of NO and PGE_2 production was concomitant with the suppression of iNOS and COX-2 expression at both the protein and mRNA levels. We also showed that this inhibition is not associated with changes in NF-κB DNA-binding activity.

The effects of HDAC inhibition on the production of NO and the expression of iNOS have been examined in a few recent studies. Yu *et al.* demonstrated that TSA inhibited

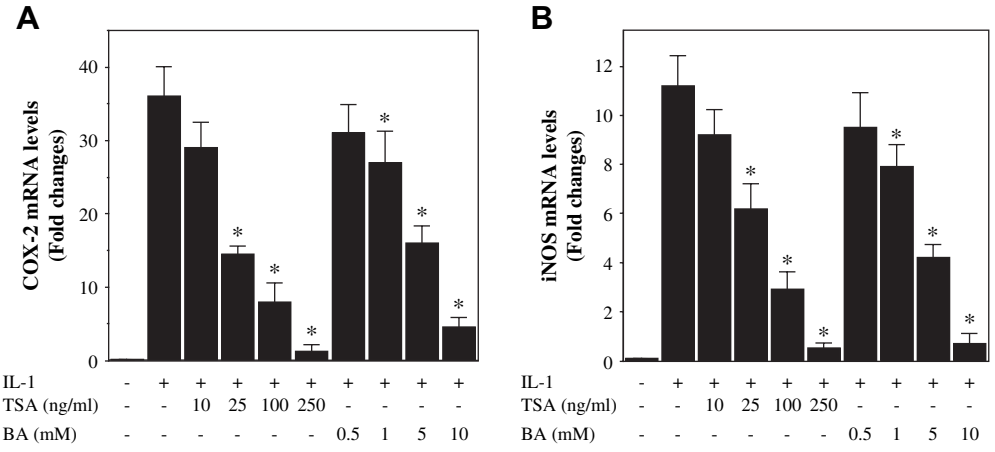

Fig. 5. TSA and BA decrease IL-1-induced iNOS and COX-2 mRNA expression. Chondrocytes were stimulated with 100 pg/ml IL-1 in the absence or presence of increasing concentrations of TSA or BA for 6 h. Total RNA was isolated, cDNA was synthesized; and iNOS and COX-2 mRNAs were quantified using real-time PCR. GAPDH gene expression was used for normalization. The results are expressed as − fold changes considering one as the value of untreated cells. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. The results are expressed as mean ± S.E.M. of four independent experiments. *$P < 0.05$ compared with cells treated with IL-1 alone (control).

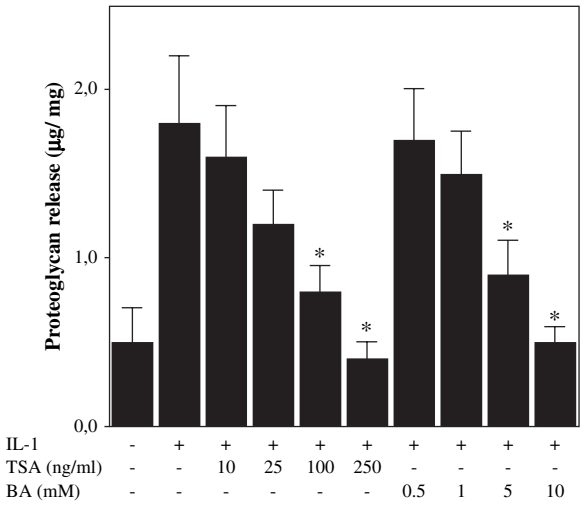

Fig. 6. TSA and BA suppress IL-1-induced cartilage proteoglycan degradation. Cartilage explants were stimulated with 1 ng/ml IL-1 in the absence or presence of increasing concentrations of TSA or BA for 72 h. Proteoglycan degradation was assessed by assaying aliquots of culture media for GAG release. Results are expressed as μg GAG/mg cartilage and are the mean ± S.E.M. from four independent experiments. $^*P < 0.05$ compared with cells treated with IL-1 alone.

IL-1- or LPS + IFN-γ-induced NO production in mesangial cells and RAW 264.7 cells[41]. Leoni *et al.*[29] showed that another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), dose-dependently attenuated NO release from mouse peritoneal macrophages stimulated by the combination of TNF-α and IFN-γ. Similarly, Larsen *et al.*[42] found that TSA and SAHA prevented the production of NO and the expression of iNOS in the β-cell line INS-1 and in intact rat islets treated with IL-1 + IFN-γ[42] On the other hand, TSA and SAHA have been shown to enhance LPS-induced production of NO in microglial cells[43], and butyrate was reported to increase the expression of iNOS and the production of NO in response to treatment with LPS + IFN-γ in intestinal epithelial cells[44]. The reasons for these discrepancies are unclear but may be attributable to several factors including cell type and stimulation conditions. We also demonstrated that TSA and BA prevented IL-1-induced PGE_2 release in chondrocytes. Furthermore, TSA and BA suppressed IL-1-induced COX-2 expression at the mRNA and protein levels. These findings are in agreement with previous studies showing that HDAC inhibitors prevented the induction of PGE_2 production and COX-2 expression in several cell types[45,46].

Pro-inflammatory cytokines TNF-α and IL-17 are also believed to contribute to the pathogenesis of OA and are strong inducers of NO and PGE_2 synthesis in chondrocytes[1–3]. Interestingly, HDAC inhibitors blocked the production of NO and PGE_2 in chondrocytes treated with either TNF-α or IL-17. Thus, the suppression of NO and PGE_2 synthesis by HDAC inhibitors is not specific to IL-1 and is independent of the nature of the stimulus that triggers NO and PGE_2 production.

Several studies have demonstrated that HDAC inhibitors suppress the production of a number of pro-inflammatory cytokines *in vitro* and *in vivo*. For example, treatment with SAHA or ITF2357 decreased the release of IL-1, IL-12, TNF-α and IFN-γ from LPS-stimulated human peripheral blood mononuclear cells[29,30]. TSA treatment was also reported to prevent the expression of IL-8 in Caco-2 cells[47] and of IL-12 in lung epithelial cells stimulated with LPS[48]. *In vivo*, SAHA dose-dependently reduced the circulating levels of the pro-inflammatory cytokines TNF-α, IL-1, and IL-6 in an endotoxemia model[29]. In addition to their anti-inflammatory effects, HDAC inhibitors display

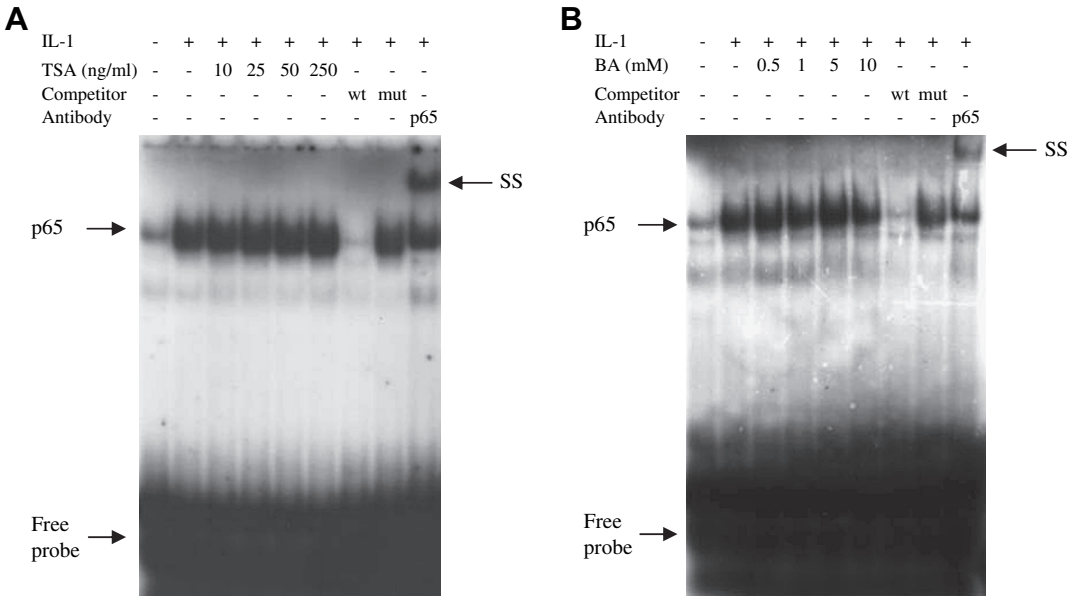

Fig. 7. Effect of TSA and BA on DNA-binding activity of NF-κB. Confluent chondrocytes were treated with IL-1 (100 pg/ml) in the absence or presence of increasing concentrations of TSA (A) or BA (B) for 1 h. Nuclear extracts were prepared and incubated with a ^{32}[P]-labeled oligonucleotide containing the NF-κB sequence. Specificity of binding was confirmed using 50-molar excess of wild-type and mutated unlabeled oligonucleotides. The supershifted (SS) band is indicated. The autoradiograph shown is representative of similar results obtained from four independent experiments.

chondroprotective properties. Indeed, we demonstrated here that treatment with TSA or BA prevents IL-1-induced proteoglycan degradation in cartilage explants. Moreover, Young *et al.*[49] showed that HDAC inhibitors blocked the induction of several enzymes responsible for cartilage degradation, including MMP-1, MMP-13, a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS) -4, -5 and -9 and prevented cartilage degradation in an explant assay[49]. Together these data suggest that HDAC inhibitors may prevent cartilage destruction in arthritis. Indeed, HDAC inhibitors prevent cartilage damage in models of adjuvant-induced arthritis[50] and autoantibody-mediated arthritis[51]. Protective effects of HDAC inhibitors on cartilage were also observed in collagen-induced arthritis models[52].

The transcription factor NF-κB is important in the induction of iNOS and COX-2 by pro-inflammatory cytokines and stimuli in chondrocytes, and the 5′-flanking regions of both *iNOS* and *COX-2* genes contain binding sites for NF-κB[39,40]. In the present study, we demonstrated that IL-1 enhances the binding activity of NF-κB p65. Interestingly, treatment with TSA or butyrate did not affect the binding activity of NF-κB, suggesting that HDAC inhibitors influence NF-κB-dependent gene expression down-stream of DNA-binding in chondrocytes. These results are in accordance with previous reports showing that HDAC inhibitors did not affect the DNA-binding activity of NF-κB in IL-1-stimulated mesangial cells[41], and Caco-2 cells[47], as well as in LPS-stimulated N9 microglia cells[43]. In contrast, other groups have reported that HDAC inhibitors reduced the DNA-binding activity of NF-κB in A549 cells[53] and human colon cell lines[54] treated with pro-inflammatory cytokines. Several reasons may explain this dichotomy including the differences in time exposure to HDAC inhibitors and the model used.

There are a number of potential mechanisms by which HDAC inhibitors could inhibit IL-1-induced iNOS and COX-2 expression. First, HDAC inhibitors may down-regulate gene expression by altering local chromatin structure secondary to increased histone acetylation. Secondly, the suppressive effect of HDAC inhibitors could be mediated by hyperacetylation of transcription factors or signaling molecules that participate in IL-1-induced iNOS and COX-2 expression. Finally, gene products induced by HDAC inhibitors may also interfere with the signaling pathways involved in iNOS and COX-2 expression. Regardless of the exact mechanism by which HDAC inhibitors down-regulate IL-1-induced NO and PGE_2 production, these results are very interesting from a pharmacological point of view since inhibitors of PGE_2 and NO production are a promising class of compounds with therapeutic potential for OA.

In conclusion, we have shown that HDAC inhibitors suppress IL-1-induced NO and PGE_2 production, iNOS and COX-2 expression as well as proteoglycan degradation. The mechanism by which HDAC inhibitors attenuate IL-1-effects is independent of the DNA-binding activity of the transcription factor NF-κB. These data also suggest that HDAC inhibitors represent a promising new class of compounds in the treatment of OA.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors thank Virginia Wallis for her assistance with the manuscript preparation. This work was supported by the Canadian Institutes of Health Research (CIHR) Grant MOP-84282, and the Fonds de la Recherche du Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CHUM). HF is a Research Scholar of the Fonds de Recherche en Santé du Québec (FRSQ).

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