Journal of Cellular Biochemistry 100:1217–1231 (2007)

Differential Regulation of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase by 4-Hydroxynonenal in Human Osteoarthritic Chondrocytes Through ATF-2/CREB-1 Transactivation and Concomitant Inhibition of NF-κB Signaling Cascade

France Vaillancourt, Barbara Morquette, Qin Shi, Hassan Fahmi, Patrick Lavigne, John A. Di Battista, Julio C. Fernandes, and Mohamed Benderdour*

Orthopaedic Research Laboratory, Sacre-Cœur Hospital, University of Montreal, Montreal, Quebec, Canada

Abstract 4-hydroxynonenal (HNE), a lipid peroxidation end product, is produced abundantly in osteoarthritic (OA) articular tissues and was recently identified as a potent catabolic factor in OA cartilage. In this study, we provide additional evidence that HNE acts as an inflammatory mediator by elucidating the signaling cascades targeted in OA chondrocytes leading to cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) gene expression. HNE induced COX-2 protein and mRNA levels with accompanying increases in prostaglandin E2 (PGE_2) production. In contrast, HNE had no effect on basal iNOS expression or nitric oxide (NO) release. However, HNE strongly inhibited IL-1β-induced iNOS or NO production. Transient transfection experiments revealed that the ATF/CRE site (−58/−53) is essential for HNE-induced COX-2 promoter activation and indeed HNE induced ATF-2 and CREB-1 phosphorylation as well as ATF/CRE binding activity. Overexpression of p38 MAPK enhanced the HNE-induced ATF/CRE luciferase reporter plasmid activation, COX-2 synthesis and promoter activity. HNE abrogated IL-1β-induced iNOS expression and promoter activity mainly through NF-κB site (−5,817/−5,808) possibly via suppression of IKKα-induced IκBα phosphorylation and NF-κB/p65 nuclear translocation. Upon examination of upstream signaling components, we found that IKKα was inactivated through HNE/IKKα adduct formation. Taken together, these findings illustrate the central role played by HNE in the regulation of COX-2 and iNOS in OA. The aldehyde induced selectively COX-2 expression via ATF/CRE activation and inhibited iNOS via IKKα inactivation. J. Cell. Biochem. 100: 1217–1231, 2007.

Key words: osteoarthritis; inflammation; COX-2; iNOS; lipid peroxidation; 4-hydroxynonenal

Osteoarthritis (OA) is a chronic joint disease characterized by progressive degenerative changes in the composition, structure, and function of articular tissues. The pathogenesis of OA has been associated with the presence of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNFα). Both of these mediators can induce the production and release of reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin E_2 (PGE_2) [Jang and Murrell, 1998; Hashimoto et al., 1999; Hughes et al., 1999; LeGrand et al., 2001; Hardy et al., 2002; Brenner et al., 2004]. Data from experimental and clinical models showed that these chemical mediators play a pivotal role in the pathogenesis of OA mainly by contributing in the inflammatory and catabolic process [Amin et al., 1997; Amin and Abramson, 1998; Clancy et al., 1998; Hashimoto et al., 1999; Hughes et al., 1999]. PGE_2 and NO are synthesized mostly by cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) respectively. COX-2 and iNOS are an early response genes

Grant sponsor: The Arthritis Society; Grant sponsor: Pfizer Canada; Grant number: NRA3190022.

*Correspondence to: Dr. Mohamed Benderdour, Laboratoire de Recherche en Orthopédie, Hôpital du Sacré-Cœur porte, K-3045, 5400 boul. Gouin Ouest, Montreal (Qc) Canada H4J 1C5.

E-mail: mohamed.benderdour@umontreal.ca

Received 5 June 2006; Accepted 24 July 2006

DOI 10.1002/jcb.21110

that are rapidly induced often by IL-1β and TNFα and most likely responsible for the elevated PGE_2 and NO levels in OA [Shalom-Barak et al., 1998; Amin et al., 1999; Martel-Pelletier et al., 2003].

The molecular signaling mechanisms that lead to the induction of COX-2 and iNOS is complex and varies according to the cell type and the stimulus applied. One of the most extensively investigated intracellular signaling cascades involved in proinflammatory responses is the MAPK pathway. Three distinct groups of well-characterized major MAPK subfamily members include extracellular-regulated protein kinase (ERK), c-Jun NH_2-protein kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAPK that are serine/threonine protein kinases. In chondrocytes, several lines of evidence showed that IL-1β-induced COX-2 gene expression and PGE_2 production were controlled by NF-IL-6, ATF-2, and CREB-1 transcription factors, a process associated with activation of p38 MAPK, JNK/SAPK, and ERKs [Sorli et al., 1998; Faour et al., 2003; McEvoy et al., 2004; Nieminen et al., 2005]. However, the known main regulator of iNOS gene expression and NO production is the transcription factor NF-κB, a DNA-binding protein important for regulation of various genes involved in immune and inflammatory responses [Taylor et al., 1998; Mendes et al., 2002; Singh et al., 2002]. Activation of NF-κB requires ubiquitin-dependent degradation of the corresponding inhibitory proteins IκB that retain inactive NF-κB in the cytosol [Thanos and Maniatis, 1995; Mercurio et al., 1997]. Cytokines lead to the phosphorylation of IκB by IκB kinase (IKK) at two serine residues at its N-terminus, and phosphorylated IκB is then removed from the NF-κB complex, allowing the translocation of NF-κB into the nucleus [Traenckner et al., 1995; Mercurio et al., 1997].

Lipid peroxidation (LPO) is a free radical-related process occurring in biologic systems under nonenzymatic control with the generation of lipid-derived aldehydes [Halliwell and Chirico, 1993]. This involves oxidative decomposition of ω-3 and ω-6 polyunsaturated fatty acids and membrane phospholipids, leading to the formation of complex mixtures of lipid hydroperoxide and aldehydic end-products. Several reports have shown that lipo-polysaccharide (LPS), IL-1β and TNFα are able to induce the formation of aldehydes both in vivo and in vitro [Bohler et al., 2000; Kimura et al., 2005]. Similar to ROS, aldehydes are electrophiles that bind to the nucleophilic groups of proteins, (amino)phospholipids and nucleic acids. However, their relatively longer half-life compared to free-radicals make them candidates for the propagation of damage to neighboring cells. One of the most important products of LPO is 4-hydroxynonenal (HNE) [Esterbauer et al., 1991]. This aldehyde is cytotoxic and is generated during various physiological and pathophysiological conditions based on the production of ROS [Uchida, 2003]. Classically, HNE was described for a long time as a marker of extensive oxidative stress in various tissues. Recent studies, however, have showed that HNE can modulate cellular metabolism, inflammatory responses, as well as apoptosis via its effects on signaling/transcription regulation and protein modification [Uchida et al., 1999; Chen et al., 2001; Benderdour et al., 2003; Leonarduzzi et al., 2004]. Interestingly, these pathological processes are all implicated to various degrees in OA, but no study has specifically reported the potential role of HNE in this disease. In previous studies, it has been demonstrated that HNE induced COX-2 expression and inhibited iNOS in various cell lines [Kumagai et al., 2000; Hattori et al., 2001; Liu et al., 2001]. These data lead us to believe that HNE could play an important role in the modulation of the inflammatory response in OA. Recently, we have reported that HNE level in synovial fluids of OA patients was higher compared to normal subjects [Morquette et al., 2006]. We therefore provided strong evidence of the catabolic role of HNE in OA cartilage by its ability to induce transcriptional and posttranslational modification of type II collagen (C II) and MMP-13. With its reactivity to proteins, HNE was shown to activate MMP-13 and accelerate C II degradation.

The objective of this study was to further elucidate the molecular mechanism underlying HNE-mediated COX-2 and iNOS expression in OA chondrocytes as well as the production of their respective metabolic end-products, namely PGE_2 and NO. In the present study, we have identified two distinct mechanisms of action of HNE in OA chondrocytes, which include upregulation of COX-2 via ATF/CRE activation and inhibition of iNOS via NF-κB inactivation.

MATERIALS AND METHODS

Specimen Selection and Chondrocytes Culture

Cartilage specimens were obtained from patients with OA (64 ± 8 years, mean $\pm$ SEM) who underwent total knee arthroplasty ($n = 24$). Diagnosis was established according to the American College of Rheumatology criteria (Altman et al., 1986). OA cartilage (femoral condyles and tibial plateaus) was obtained under aseptic conditions and carefully dissected from the underlying bone in each specimen. The experimental protocol was approved by the Research Ethics board of the Sacre-cœur hospital of Montreal. OA chondrocytes were released from sequential enzymatic digestion with 1 mg/ml of pronase (Sigma, Oakville, ON, Canada) for 1 h, followed by 6 h with 2 mg/ml of type IV collagenase (Sigma) at 37°C in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen life technology, Inc.). Isolated chondrocytes were seeded at high density in culture flasks until confluence in 10% FBS/DMEM at 37°C in a humidified atmosphere (5% CO_2/95% air). First passage chondrocytes were seeded at 10^5 cells/cm^2 in tissue culture plates. Twenty-four hours prior to experiment, the medium was replaced by a fresh medium containing 1% FBS and experiments performed in this medium supplemented with the factors under study.

PGE_2 and NO Determination

Osteoarthritis chondrocytes were incubated for 16 h at 37°C with increasing concentrations (0–20 μM) of HNE (Cayman chemical, Ann Arbor, MI) or with 10 μM HNE for increasing periods of time (0–24 h) with or without 0.1 ng/ml IL-1β (R&D systems, Minneapolis, MN). Following incubation, the culture medium was collected and nitrite (NO_2^-), a stable end product of NO, was measured in the supernatant using a spectrophotometric method based on the Griess reaction [Green et al., 1982]. PGE_2 levels were determined using a specific commercial kit (Cayman Chemical) according to the manufacturer's specifications. The sensitivity of the PGE_2 assay was 9 pg/ml.

COX-2 and iNOS Detection by Western Blotting

Twenty micrograms of total proteins from chondrocyte lysates treated under indicated conditions were subjected to discontinuous 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS–PAGE). Protein transfer, immunodetection, and semiquantitative measurements were performed as described previously [Morquette et al., 2006]. Rabbit anti-human iNOS (Cayman), rabbit anti-human COX-2 (Calbiochem EDM Biosciences, Inc., San Diego, CA), and goat anti-rabbit IgG-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used.

Transcription Factors Determinations

Experiments were conducted in OA chondrocytes treated with 10 μM HNE for increasing incubation times (0–180 min), or with increasing concentrations of HNE (0–20 μM) for 15 min in the presence of 0.1 ng/ml IL-1β. Levels of total and phosphorylated transcription factors were detected for the ATF-2, CREB-1 by Western blotting using specific rabbit antibodies (New England Biolabs, Beverly, MA). For total nuclear and cytosolic NF-κB/p65 and phosphorylated IκBα, chondrocytes were gently scraped into ice-cold PBS and pelleted by brief centrifugation. The cellular pellet was carefully resuspended in an ice-cold hypotonic lysis buffer containing 10 mM HEPES–KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 1% NP-40. Cells were allowed to swell on ice and cytosolic extracts and nuclei were recovered by brief centrifugation. The nuclear pellets were resuspended in a high-salt extraction buffer containing 20 mM HEPES–KOH (pH 7.9), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM EDTA, 25% glycerol supplemented with protease inhibitors cocktail followed by incubation on ice for 20 min. Ten micrograms of nuclear and cytosolic protein were subjected to Western blotting analysis by the use of specific rabbit antibodies anti-NF-κB/p65 and anti-phospho IκBα.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

mRNA levels of COX-2, iNOS and GAPDH were analyzed semiquantitatively with a programmable thermal controller (Waterman, Biometra GmbH, Germany) as previously described [Morquette et al., 2006]. The following sense- and antisense-specific primers were

used: human COX-2 sense 5′-AAGACAGATCATAAGCGAGGGCCA-3′ and antisense 5′-TGGCATACATCATCAGACCAGGCA-3′, human iNOS sense 5′-ACATCAGGTGGGCCATTACTGTGT-3′ and antisense 5′-ATGGTCACCTCCAGCACAAGATCA-3′, human GAPDH sense 5′-CAGAACATCATCCCTGCCTCT-3′ and antisense 5′-GCTTGACAAAGTGGTCGTTCAG-3′ (BioCorp, Inc., Montreal, QC, Canada). Negative controls for RT-PCR without template RNA were included in each experiment (data not shown). PCR products were separated on 2.2% agarose gel and scanned. cDNA values for COX-2 and iNOS were normalized to that of GAPDH.

Plasmids and Transient Transfection

The human COX-2 promoter constructs used included a wild-type (WT) (−415)-Luciferase (Luc) COX-2, mutated ATF/CRE (−58/−53) (−415)-Luc COX-2, and mutated NF-κB (−223/−214) (−415)-Luc COX-2 promoter plasmid, as previously described [Faour et al., 2003]. Human WT (−7,200)-Luc iNOS and mutated NF-κB (−5,817/−5,808) (−7,200)-Luc iNOS promoter plasmid were a gift from Dr. R.A. Shapiro (University of Pittsburgh, Pittsburgh, PA). Expression vector for WT pCMV-Flag-p38 and dominant negative (DN) pCMV-Flag-p38 MAPK were a kind gift from Dr. R.J. Davies (University of Massachusetts, Worcester, MA). Expression vector for IKKα was gifted generously by Dr. M. Karin (University of California, San Diego, La Jolla, CA). The pATFCRE-1-luciferase plasmid containing four copies of ATF/CRE consensus sequences (pATF/CRE-Luc), the pNF-κB-luciferase plasmid containing five copies of NF-κB consensus sequences (pNF-κB Luc) were obtained from Stratagene (La Jolla, CA). A pCMV-β-galactosidase (pCMV-β-gal) reporter vector was purchased from Promega (Madison, WI).

Subconfluent human OA chondrocytes were transiently transfected in 12-well cluster plates using lipofectamine 2000^{TM} reagents methods (Invitrogen life technology) according to the manufacturer's protocol. Briefly, transfections were conducted for 6 h with DNA lipofectamine complexes containing 10 μl of lipofectamine reagent, 2 μg DNA plasmid and 0.5 μg of pCMV-β-gal (as a control of transfection efficiency). After washing, experiments were performed in 1% FBS fresh medium supplemented with the factors under study. For COX-2 and iNOS promoter study, luciferase activity was determined in cellular extracts using a commercial kit (Luciferase Assay System; Promega). Data were normalized to β-gal level, which was quantified by a specific Elisa assay (Roche Applied Science, Laval, QC Canada). The effect of WT p38 MAPK and IKKα overexpression on COX-2 and iNOS expression was evaluated by determining the promoter activity as well as protein level of COX-2 and iNOS as described previously in the presence or absence of 10 μM p38 MAPK inhibitor SB202190 or 5 μM of IKK inhibitor Bay 11−7082 (Calbiochem).

Electrophoretic Mobility Shift Analysis (EMSA)

Osteoarthritis chondrocytes were incubated for 1 h with or without increasing concentration of HNE (0−20 μM) in the presence or absence of 0.1 ng/ml IL-1β. Nuclear proteins were extracted as described above. Double-stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP (Amersham Biosciences, Baie-d'Urfé, Qc, Canada) using T4 polynucleotide kinase (Promega). The sense sequences of the oligonucleotides tested were as follows: ATF/CRE (COX-2), 5′-CAG TCA TTT CGT **CA**C ATG GGC TTG-3′, NF-κB, 5′-AGT GGG GAC TA**C C**CC CTC TGC-3′. 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 of poly(dI-dC). The binding reactions were conducted with 10 μg of nuclear extract and of 200,000 cpm of [γ-^{32}P]-labeled oligonucleotide probe at 22°C for 20 min. Specificity of the binding was assayed by competition of the oligonucleotide with 20-fold of excess unlabeled WT oligonucleotide or mutated (mu) unlabeled oligonucleotide. The binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis in a Tris-borate-EDTA buffer, after which the gels were fixed, dried, and exposed to Kodak X-AR5 films.

Immunoprecipitation and Kinase Assay for IKKα

For HNE/IKKα adduct detection, OA chondrocytes ($\sim 4 \times 10^6$ cells) were preincubated with 0.1 ng/ml IL-1β for 30 min followed by another incubation for 30 min with either 10 or 20 μM HNE. Then, cells were lysed on ice in 1 ml kinase lysis buffer (KLB: 40 mM Tris (pH 8.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na_3VO_4, 1 mM DTT)

supplemented with protease inhibitors cocktail as described by Ji et al. [2001]. One hundred micrograms of total protein were subjected to immunoprecipitation with rabbit anti-IKKα antibody (1:500; New England Biolabs) in KLB buffer containing 0.5 M NaCl for overnight at 4°C and then for an additional 2 h with protein A (Santa Cruz Biotechnology, Santa Cruz, CA). The resin was washed with KLB buffer and proteins were analyzed by Western blotting using rabbit anti-HNE antibody (1:1,000; Calbiochem). For the IKKα assay, the washed resin were incubated with 20 μl of kinase buffer containing 2 μg of GST-IκBα substrate (Santa Cruz Biotechnology), 50 μM ATP, and 2 μCi of [γ-^{32}P]ATP (Amersham Biosciences) for 30 min at 30°C. The proteins were resolved by 4–12% discontinuous SDS–PAGE, transferred, and the membranes were exposed to Kodak X-AR5 films.

Statistical Analysis

The data are expressed as the mean ± SEM. Statistical significance was assessed by Student's unpaired *t*-test and *P*-values less then 0.05 were considered significant.

RESULTS

HNE Induces PGE_2 Production but not NO

Dose-response and time-course experiments were first performed to explore the effect of HNE on PGE_2 and NO production by OA chondrocytes. As shown in Figure 1, HNE induced PGE_2 production and this induction was dose (Fig. 1A) and time-dependent (Fig. 1B). Combined with 0.1 ng/ml IL-1β, HNE reduced slightly but not significantly IL-1β-induced PGE_2 production. In contrast, HNE at different concentration (Fig. 1C) and at different time of incubation (Fig. 1D) had no effects on basal NO production. However, IL-1β-induced NO production was significantly reduced with the addition of HNE. Of note, chondrocytes treatment with HNE up to 20 μM did not alter the cellular viability, but at 50 μM, HNE was found to be cytotoxic and decreased significantly the cell viability about 50% (data not shown).

HNE Induces COX-2 Synthesis

To better characterize the properties of HNE cell signaling, we performed both dose-response and time-course studies on the induction of the

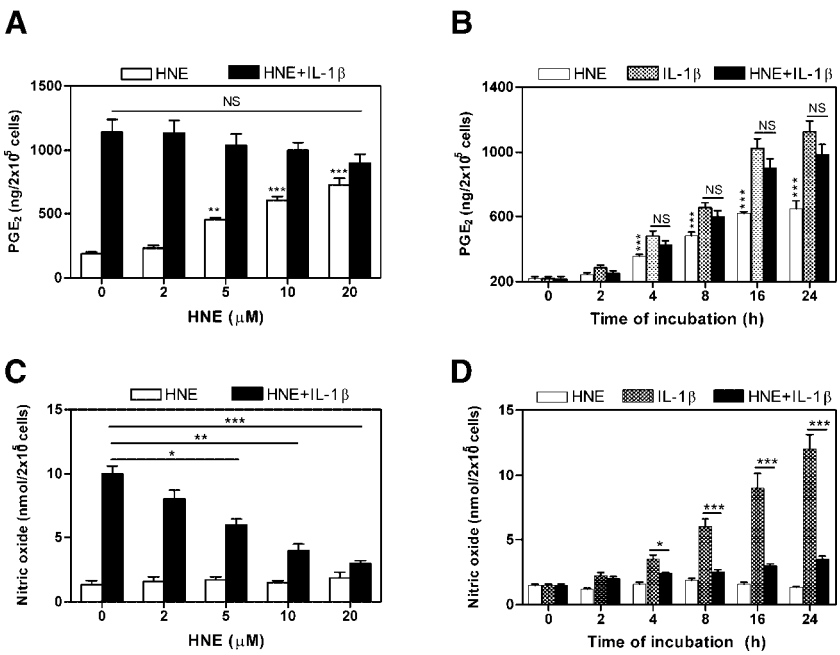

Fig. 1. Dose and time related effects of HNE on PGE_2 and NO production in OA chondrocytes. OA chondrocytes were treated for 16 h with increasing concentration of HNE (0–20 μM) (**A**, **C**) or with 10 μM HNE for increasing periods of time (**B**, **D**) in the presence or absence of 0.1 ng/ml IL-1β. Levels of PGE_2 (A, B) and NO (C, D) were determined in culture medium using commercial kits or using a spectrophotometric method based on the Griess reaction respectively. Data are mean ± SEM. of n = 5 experiments. Statistics: Student's unpaired *t*-test; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistical significance was obtained when basal level was compared to HNE treatment and when IL-1β-treatment was compared to HNE+ IL-1β. NS (not significant).

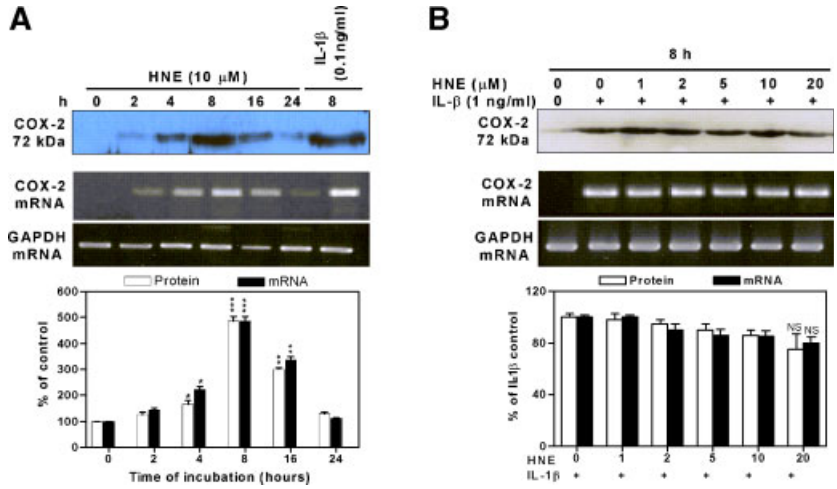

Fig. 2. Effects of HNE and combining IL-1β and HNE on COX-2 expression. **A**: OA chondrocytes were incubated with 10 μM HNE for increasing periods of time (0–24 h) or with 0.1 ng/ml IL-1β for 8 h. **B**: OA chondrocytes were co-stimulated with 0.1 ng/ml IL-1β and increasing concentration of HNE (1–20 μM) for 8 h. COX-2 expression at protein and mRNA levels was determined respectively by western blotting and RT-PCR assay as described in ''Materials and Methods.'' Data are mean ± SEM of n = 3. Statistics: Student's unpaired *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical significance was obtained when basal level was compared to HNE treatment and when IL-1β-treatment was compared to HNE+ IL-1β. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

COX-2 by HNE in the absence or presence of 0.1 ng/ml IL-1β. Our data showed that COX-2 expression at protein and mRNA levels was detectable within 2 h of incubation with 10 μM of HNE and plateaued at 8 h of incubation before declining afterwards (Fig. 2A). Combined with IL-1β, HNE at different concentrations had no effect on IL-1β-induced COX-2 after 8 h of incubation (Fig. 2B).

HNE Inhibits iNOS Synthesis

In order to determine that NO inhibition by HNE treatment is related to iNOS reduction, we have studied both the effects of increasing dose of HNE and time of incubation on iNOS expression. In contrast to IL-1β, treatments of chondrocytes with 10 μM HNE had no effect on basal iNOS expression at protein and mRNA levels (Fig. 3A). However, the IL-1β-induced iNOS expression was gradually reduced by HNE in a dose-dependent manner after 8 h of incubation (Fig. 3B).

HNE Modulates COX-2 and iNOS Promoter Activities

To gain insight into the mechanism of HNE mediated COX-2 and iNOS production at the transcriptional level, OA chondrocytes were transiently transfected with COX-2 and iNOS promoter constructs. Compared to unstimulated transfected cells, our data (Fig. 4A) showed that 10 μM HNE as well as 0.1 ng/ml IL-1β upregulated the activity of WT (−415)-Luc COX-2 promoter construct by 1.6- and 1.8-fold respectively. Combined with 0.1 ng/ml IL-1β, HNE reduced slightly but not significantly the promoter activity. To examine for elements of transcriptional control of the COX-2 gene by HNE, we conducted further transient transfection with mutated ATF/CRE or NF-κB (−415)-Luc COX-2 promoter constructs. Data (Fig. 4A) revealed that inactivation of the ATF/CRE (−58/−53) site resulted in a decrease of over 75% of the basal WT COX-2 promoter activity. In addition, their inducibility by either HNE or IL-1β was completely abrogated. However, mutating the more proximal NF-κB site (−223/−214) in the human COX-2 promoter construct was without effect in terms of basal and HNE-stimulated promoter activity.

In contrast to COX-2, iNOS promoter studies showed that IL-1β (0.1 ng/ml) but not HNE (10 μM) induced the WT (−7,200)-Luc iNOS promoter activity by 1.7-fold (Fig. 4B). The IL-1β-induced iNOS promoter activity was completely abrogated by HNE. To determine the role of NF-κB in HNE mediating iNOS expression, we conducted transient transfection with the mutated NF-κB (−5,817/5,808) (−7200)-Luc iNOS promoter construct. Our data showed that the mutation of NF-κB site results in loss of

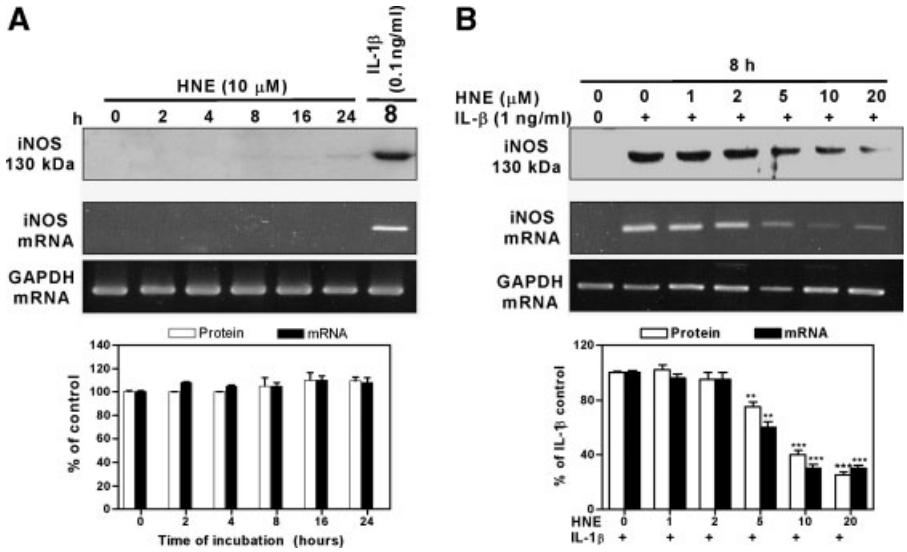

Fig. 3. Effects of HNE and combining IL-1β and HNE on iNOS expression. **A**: OA chondrocytes were incubated with 10 μM HNE for increasing periods of time (0–24 h) or with 0.1 ng/ml IL-1β for 8 h. **B**: OA chondrocytes were co-stimulated with 0.1 ng/ml IL-1β and increasing concentration of HNE (1–20 μM) for 8 h. iNOS expression at protein and mRNA levels were determined respectively by Western blotting and RT-PCR assay as described in ''Materials and Methods.'' Data are expressed as means ± SEM of n = 3. Statistics: Student's unpaired *t*-test; $^{**}P < 0.01$, $^{***}P < 0.001$. Statistical significance was obtained when basal level was compared to HNE treatment and when IL-1β-treatment was compared to HNE+ IL-1β. NS (not significant).

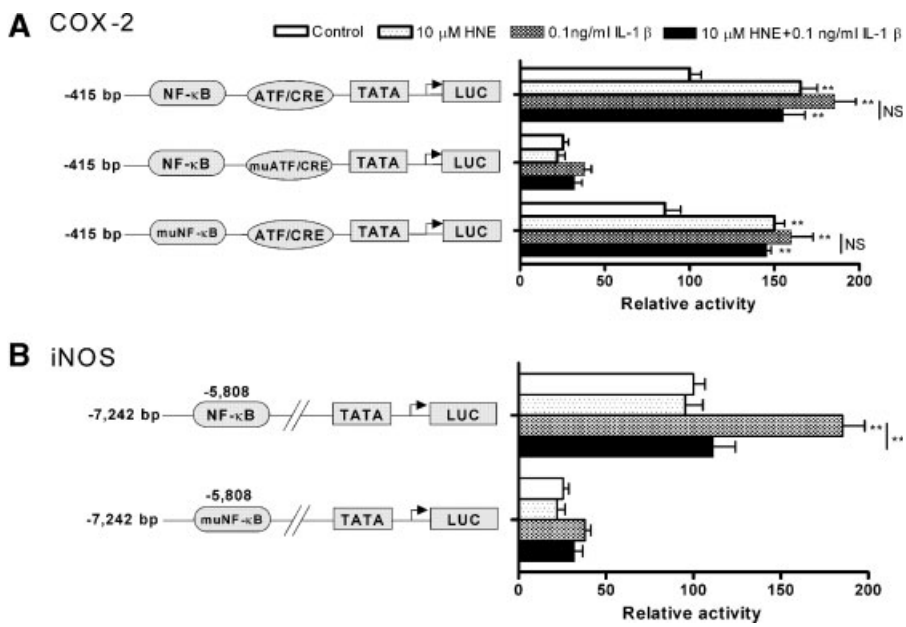

Fig. 4. Modulation of transcriptional activity of the COX-2 and iNOS promoter by HNE. **A**: One construct of the COX-2 promoter (−415 bp) fused to a Luciferase (Luc) reporter gene, and its mutated ATF/CRE (muATF/CRE) or mutated NF-κB (muNF-κB) derivatives, and (**B**) one construct of the iNOS promoter (−7.2 kb) fused to a Luc reporter gene, and its mutated NF-κB (muNF-κB) are shown in schematic (left). The constructs were co-transfected in human OA chondrocytes with pCMV-β-galactosidase (β-gal). Six hours after transfection, fresh medium with or without 10 μM HNE, 0.1 ng/ml IL-1β, or 10 μM HNE combined with 0.1 ng/ml IL-1β was added for another 24 h. The β-gal and Luc levels were then measured in chondrocyte lysates using specific ELISA, and Luc activities were normalized to that of β-gal. Values are expressed as mean ± SEM; *P*-values were determined by Student's unpaired *t*-test. $^{**}P < 0.01$. Statistical difference was obtained when unstimulated cells were compared to HNE, IL-1β, or HNE+ IL-1β-treated cells and when treated cells with IL-1β were compared to HNE+ IL-1β.

basal and IL-1β-induced promoter activity (Fig. 4B).

Transcription Factors Activation by HNE

To identify the intracellular signaling regulated by HNE and responsible for COX-2 upregulation and iNOS downregulation, chondrocytes were incubated with 10 μM HNE for increasing time (0–180 min). In a recent study, we have demonstrated that HNE activated potently p38 and faintly p44/42 and p54/46 MAPK [Morquette et al., 2006]. In Figure 5A, we showed that ATF-2 as well as CREB-1, transcription factors whose transcriptional activities are modulated by p38 MAPK, were activated by HNE in a time-dependent manner and that HNE did not affect IL-1β-induced ATF-2 or CREB-1 phosphorylation (data not shown). In addition, total ATF-2 and CREB-1 protein was not altered by HNE exposure (data not shown). In similar experiments, our data showed that HNE blocked NF-κB/p65 translocation to the nucleus (Fig. 5B). Combined with 0.1 ng/ml IL-1β, HNE prevents IL-1β-induced NF-κB/p65 translocation in the nucleus as well as IL-1β-induced IκBα phosphorylation (Fig. 5C).

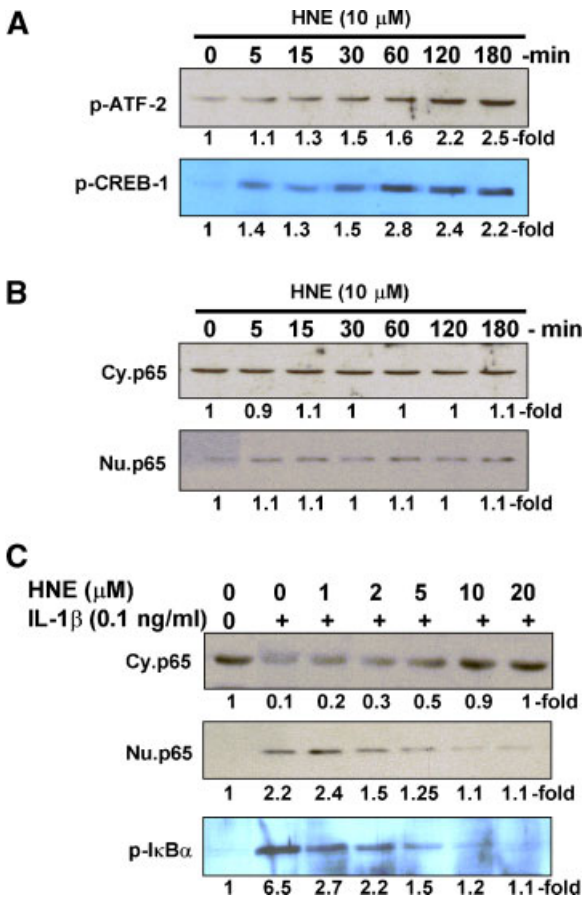

Fig. 5. Kinetics of HNE-dependent activation of ATF-2, CREB-1, NF-κB/p65, IκBα transcription factors. OA chondrocytes were incubated with 10 μM HNE for increasing periods of incubation in the presence or absence of 0.1 ng/ml IL-1β. Cellular extracts were then subjected to Western blotting using the specific polyclonal antibodies (**A**) anti-phosphoATF-2 and anti-phospho-CREB-1, (**B**) anti-NF-κB/p65, (**C**) anti-NF-κB/p65 or anti-phosphoIκBα OA as described under "Materials and Methods." [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Further, EMSA experiments using ^{32}P-labeled ATF/CRE (COX-2) oligonucleotide showed a high level of nuclear DNA-binding proteins following treatment for 1 h with increasing concentrations of HNE (Fig. 6). Confirming promoter studies, HNE stimulation of human chondrocytes produced no increases in ^{32}P-labeled NF-κB oligonucleotide binding (Fig. 6). Combined with IL-1β, HNE had no effect on IL-1β-induced ATF/CRE binding proteins but in contrast reduced dramatically IL-1β-induced NF-κB binding (Fig. 6). The specificity of the binding was confirmed by adding 20-fold excess unlabeled WT or mutated ATF/CRE and NF-κB oligonucleotides.

Effect of p38 MAPK and IKKα on HNE-Regulated COX-2 and iNOS Expression

To further confirm that the p38 MAPK pathway is implicated in HNE-induced COX-2 expression and that of IKKα implicated in HNE-inhibited iNOS, we overexpressed WT p38 MAPK and IKKα in OA chondrocytes followed by the addition of 10 μM HNE. As shown in Figure 7A, overexpression of WT p38 MAPK significantly induced COX-2 protein expression, and HNE treatment further enhanced COX-2 expression compared to control cells. To address the issue that p38 MAPK mediates HNE-induced COX-2 at transcriptional level, we conducted experiments in which OA chondrocytes were cotransfected with either p38 MAPK expression vector and WT (−415)-Luc COX-2 promoter construct. As can be seen in Figure 7B, HNE increases luciferase activity by 1.8-fold compared to control and this induction was enhanced by 3.5-fold in the presence of the presence of p38 MAPK expression vector. However, the addition 10 μM of p38 MAPK inhibitor SB202190 blocked completely this induction. Similar to p38 MAPK, overexpression of IKKα significantly augmented IL-1β-induced iNOS protein expression, whilst

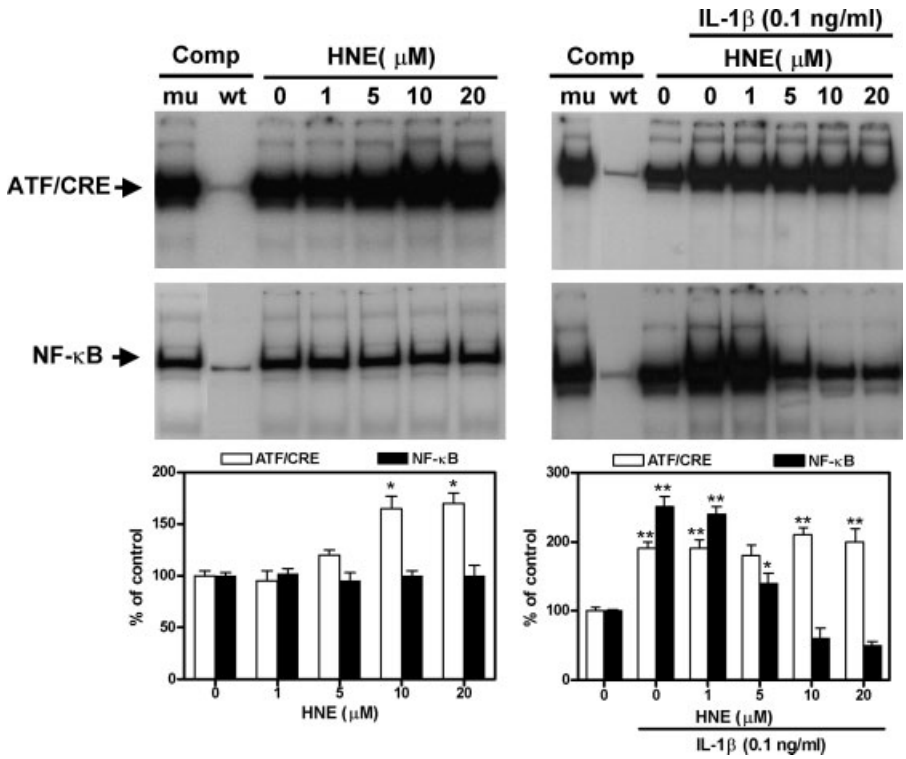

Fig. 6. Effect of HNE on ATF/CRE and NF-κB binding activity. Nuclear extracts were prepared from human OA chondrocytes treated for 1 h with increasing concentration of HNE in the presence or absence of 0.1 ng/ml IL-1β and 10 μg of protein were incubated with ^{32}P-labeled ATF/CRE (**top panel**), NF-κB (**bottom panel**) oligonucleotides for 20 min at 22°C. The binding reaction mixtures were subject to EMSA analysis as described in ''Materials and Methods.'' Specificity of the binding was assayed by competition (Comp) of the ^{32}P-labeled oligonucleotide with 20-fold of excess unlabeled ATF/CRE and NF-κB oligonucleotide or by using mutated unlabeled oligonucleotide.

increasing concentrations of HNE gradually blocked this induction (Fig. 7C). In transient transfection experiments, we showed that overexpression of IKKα enhanced TNFα-induced WT (7,200)-Luc iNOS promoter activity. This induction was suppressed by the addition of 5 μM of the IKK inhibitor Bay 11–7082 (Fig. 7D). Furthermore, the level of p38 MAPK and IKKα was very low in untransfected chondrocytes compared to transfected chondrocytes (Fig. 7A,C) and confirmed that COX-2 and iNOS protein expression was correlated respectively to p38 MAPK and IKKα expression.

HNE Regulates ATF/CRE and NF-κB via p38 MAPK and IKKα Signaling Pathways

The transcription factors ATF/CREB and NF-κB are believed to mediate the induction of a host of target genes such as COX-2 and iNOS [Taylor et al., 1998; Faour et al., 2003; McEvoy et al., 2004] and that p38 MAPK and IKKα are potent activators of ATF/CREB and NF-κB pathways respectively. We therefore, examined the influence of HNE on the transcriptional activation of the chimeric gene constructs, pNF-κB-Luc and pATF/CRE-Luc. pATF/CRE-1-Luc reporter plasmid contains four copies of the ATF/CRE response element, and pNF-κB-Luc reporter plasmid contains five copies of the NF-κB response element. In the presence of HNE (0–20 μM), we found that cotransfection with the constitutively active p38 MAPK expression vector resulted in a high induction of luciferase activity in OA chondrocytes transfected with pATF/CRE-Luc (Fig. 8A). The IL-1β induced ATF/CRE transcriptional activation was not affected by the presence of HNE. However, when cells are cotransfected with the constitutively active IKKα expression vector, we showed that HNE dose-dependently reduced the luciferase activity in transfected cells with pNF-κB-Luc (Fig. 8B). The IL-1β-induced IKKα-mediating transcriptional activation of NF-κB was gradually blocked by HNE addition.

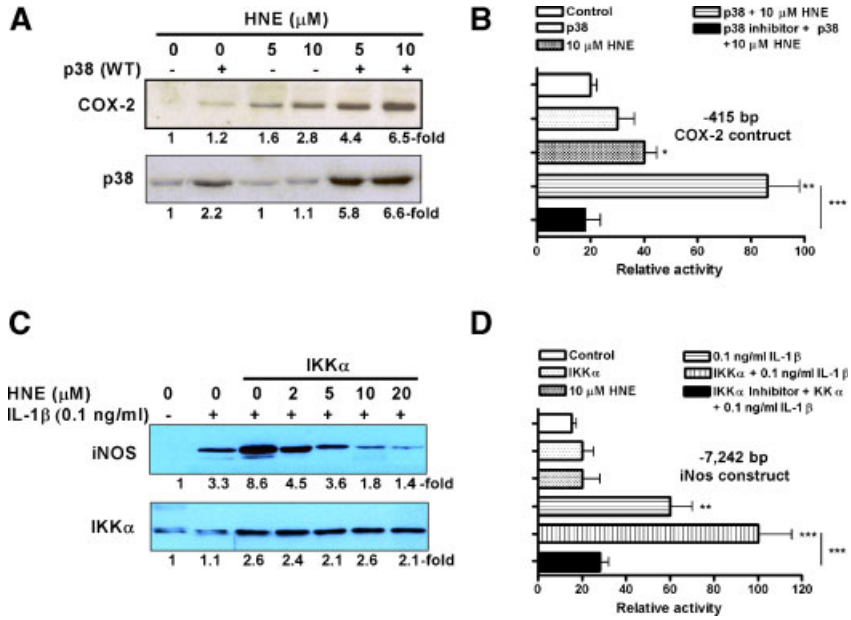

Fig. 7. Effect of p38 MAPK on HNE-induced COX-2 and IKKα on HNE-inhibited iNOS expression. Transient transfections of OA chondrocytes were performed with 1 μg of either wild type (WT) p38 MAPK expression plasmids, (−415)-Luc COX-2 promoter construct, or IKKα expression plasmids as described under ''Materials and Methods.'' Six hours following transfection, culture medium was changed and cells were co-treated with HNE (0–20 μM) and 0.1 ng/ml IL-1β for 8 h in the presence or absence of inhibitors of p38 MAPK (SB202190) or IKK (Bay 11–7082). Protein levels of (**A**) COX-2 and p38 MAPK, and (**C**) iNOS and IKKα were determined in whole cell lysates by western blotting using specific polyclonal antibodies. Promoter activity of (**B**) WT (−415)-Luc COX-2 construct, and (**D**) WT (−7,200)-Luc iNOS construct were determined using commercial kits as described previously. The results are representative of three independent experiments. Luciferase (Luc) activities were determined and normalized to β-gal and results are expressed as relative activity. Values are mean ± SEM; *P*-values were determined by Student's unpaired *t*-test. $*P < 0.05$. $**P < 0.01$. Statistical difference was obtained when basal co-transfected cells with COX-2 promoter construct and p38 MAPK were compared to HNE treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

HNE-Binding Affects IKKα Activity

HNE is an endogenous aldehyde that binds to several enzymes and modulates their activity. In an attempt to explain the decrease in IκBα phosphorylation observed in OA chondrocytes treated with HNE, we tested whether binding of HNE to IKKα could result in the inhibition of this kinase. To achieve this, HNE/IKKα adducts level were measured after incubation of chondrocytes with 0.1 ng/ml IL-1β followed by another incubation with different concentration of HNE. Cellular extracts of OA chondrocytes exposed to 10 and 20 μM HNE showed increased HNE/IKKα adducts level and a corresponding dose-dependent inhibition of IL-1β-induced IKKα activity by HNE addition as measured by IκBα phosphorylation level (Fig. 9).

DISCUSSION

HNE occupies a prominent place in the hierarchy of LPO end-products and growing evidence supports its as a (patho)physiological modulator of signal transduction, transcriptional regulation, and post-translational modification of proteins. The relevance of HNE to joint biology and pathology is now becoming clearer. In our previous study, we have reported for the first time that HNE, a highly reactive LPO product, was significantly increased in synovial fluids of patients with OA and in OA chondrocytes treated with ROS donors. We demonstrated that HNE induced transcriptional and post-translational modification of C II and MMP-13, suggesting that HNE could play a role in cartilage degradation in OA [Morquette et al., 2006]. In the present study, we have evaluated the potential role of HNE in OA inflammatory response. With the ultimate goal of clarifying this role, we documented the ability of HNE to modulate COX-2 and iNOS as well as the production of their respective end-products, PGE_2 and NO. To date there is no data demonstrating the effect of HNE on COX-2 and iNOS expression in OA articular tissues.

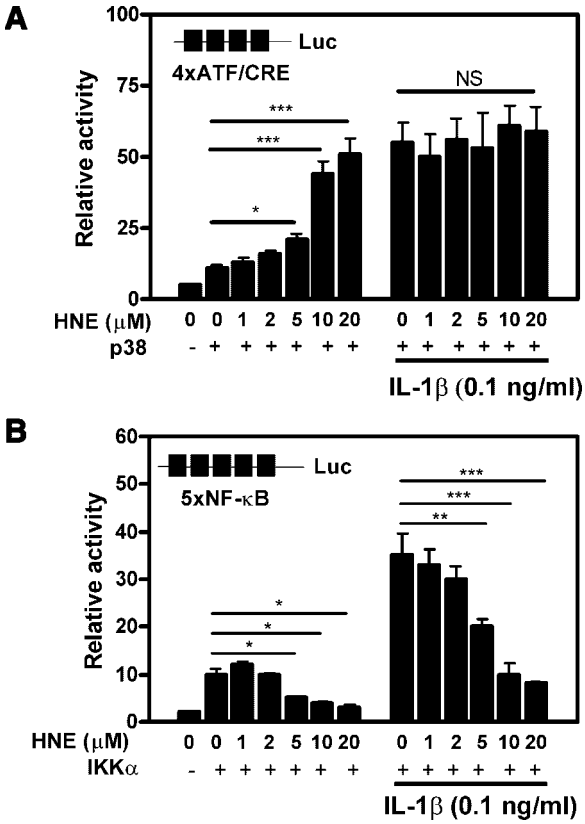

Fig. 8. HNE modulates p38 MAPK-induced CRE/ATF activation and IKKα-induced NF-κB activation. OA chondrocytes were transiently cotransfected with 1 μg of either (**A**) pCRE/ATF-Luc plasmid containing four copies of the consensus ATF/CRE responses elements (4× ATF/CRE) and p38 MAPK expression plasmid, or (**B**) pNF-κB-Luc plasmid containing 5 copies of the consensus NF-κB responses elements (5× NF-κB) and IKKα expression plasmid, together with 1 μg of pCMV-β-galactosidase (β-gal). Six h following transfection, cells were treated with increasing concentration of HNE in the presence or absence of 0.1 ng/ml IL-1β for 24 h. Luciferase (Luc) activity was determined and normalized to β-gal and results are expressed as relative activity. Values are expressed as mean ± SEM; *P*-values were determined by Student's unpaired *t*-test. * $P < 0.05$., ** $P < 0.01$, *** $P < 0.001$. Statistical difference was obtained when basal transfected cells with p38 MAPK or IKKα expression vector were compared to HNE treatment and when treated cells with IL-1β were compared to HNE+ IL-1β.

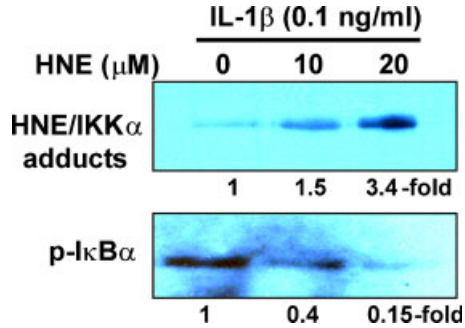

Fig. 9. Regulation of IKKα activity by HNE binding. OA chondrocytes were pre-incubated for 30 min in the presence or absence (control) of 10 or 20 μM HNE followed by another incubation for 30 min with 0.1 ng/ml IL-1β. HNE/IKKα adducts (upper) were evaluated in total protein extract subjected to immunoprecipitation using polyclonal anti-IKKα antibody and then analyzed by western blotting using polyclonal anti-HNE antibody as described in ''Materials and Methods.'' The IKKα activity (lower) was assayed in the immunoprecipitated complex using IκBα-GST fusion protein as substrate and [γ-^{32}P]ATP. Samples were then subjected to SDS–PAGE and transferred into polyvinylidene difluoride membrane before autoradiography. The results are representative of 3 independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We found that HNE increased PGE_2 release by OA chondrocytes in a dose-dependent and time-related manner. Moreover, our data showed that HNE-induced COX-2 production at protein and mRNA levels required the activation of p38 MAPK signaling pathway. Furthermore, overexpression of WT p38 MAPK in OA chondrocytes enhanced HNE-induced COX-2 protein expression and promoter activity. These results were supported by others recent studies indicating that HNE is a potent inducer of COX-2 and PGE_2 release in various cell lines such as rat liver epithelial RL34 cells and RAW264.7 macrophages cell line [Kumagai et al., 2000, 2002, 2004]. In addition, this induction is specific for HNE since others aldehydes such as acrolein and 2-nonenal possessing an analogous functionality to HNE were all inactive in term of COX-2 expression in these cells [Kumagai et al., 2000]. It has been demonstrated that HNE induced COX-2 expression by the stabilization of COX-2 mRNA via the p38 MAPK pathway [Kumagai et al., 2002]. Inhibition of p38 MAPK signal by its specific inhibitor SB203580 decreased the HNE-induced COX-2 mRNA stability.

p38 MAPK can phosphorylate members of the ATF/CREB family, rendering them transcriptionally competent [Livingstone et al., 1995]. We have shown that HNE exposure results in increase of phosphorylated ATF-2 and CREB-1 transcription factors and ATF/CRE binding activity. Interestingly, in cotransfection experiments, we showed that HNE enhanced the activity of ATF/CRE site induced by p38 MAPK overexpression. Thus, the findings from these experiments, taken together with those from previous studies, suggest that COX-2 upregulation by HNE may, in part, be attributed to the

transcriptional activation of ATF/CREB proteins family by HNE. In addition, when HNE was combined with proinflammatory cytokines, it resulted in a slight decrease in IL-1β-induced COX-2. This suggests that there are no synergetic interactions between IL-1β and HNE. We do not have explanation for these finding and additional work will be conducted to more understand HNE signaling pathways in OA chondrocytes.

There are a number of cis-elements found in the promoter region of the COX-2 gene that may exert transcriptional control. Sequence analysis of the 5′-flanking region has shown several potential transcription regulatory sequences, including a TATA box, a C/EBP motif, two AP-2 sites, three SP-l sites, two NF-κB sites, a CRE motif and an Ets-1 site [Appleby et al., 1994]. Our data from OA chondrocyte transiently transfected with plasmid constructs containing the promoter of human COX-2 with either WT or mutated sites confirmed that ATF/CRE is a key factor for basal and HNE stimulated COX-2 production. However, mutating the more proximal NF-κB in the human COX-2 promoter construct was without any effect in the basal and HNE-stimulated luciferase activity meaning that NF-κB site in the promoter region of COX-2 gene is not involved in the HNE-induced COX-2 expression. These data are in agreement with those of Kumagai et al. [2002], who demonstrated that HNE-increased COX-2 expression does not require NF-κB signaling pathways in RL34 cells. However, it has been suggested that, under proinflammatory cytokines treatment, the stimulation of COX-2 production involves more than ATF/CRE activation alone. For example, in many cell types, NF-κB pathway is one of the key transcription factors involved in IL-1β or TNFα-induced COX-2 [Crofford et al., 1997; Schmedtje et al., 1997; Shishodia et al., 2004]. In contrast, Faour et al. [2003] have reported that basal COX-2 promoter activity was not affected when the proximal NF-κB (−223/−214) site was mutated in transfected OA human chondrocytes, nor did the mutation abrogated IL-17 induced COX-2 promoter activity. Altogether, our findings combined with the existing literature indicate that the involvement of NF-κB signaling pathways in COX-2 upregulation is cell and mediator-type dependent.

The upregulation of COX-2 by HNE contrasted with that of iNOS expression. We have documented that HNE had no effect on NO release and iNOS expression at protein and mRNA levels. Furthermore, our results showed that HNE reduced dramatically but not completely IL-1β-induced NO and iNOS production. Through a series of coordinated experiments, a link between iNOS downregulation and inactivation of the NF-κB pathway was observed. Our results showed that HNE by itself did not induce NF-κB/p65 translocation or IκBα phosphorylation. Further, we have found that HNE prevents IL-1β-induced IκBα phosphorylated and NF-κB/p65 translocation in the nuclei of chondrocytes, suggesting that the inhibitory effect of HNE on the induction of iNOS expression by IL-1β is mostly a result of NF-κB inactivation. Also, our transfection experiments with the iNOS promoter suggested that NF-κB site at −5.8 kb may be involved potentially in HNE-reduced iNOS production. Several studies have shown that the expression of iNOS is dependent on the activation of the ubiquitously expressed transcription factor NF-κB. It has been reported that the human iNOS promoter contains several potential transcription factor-binding sites, including five potential NF-κB binding sites localized from −4.7 to −7.2 kb of iNOS promoter. Four were shown to be functional by mutational analysis [Taylor et al., 1998]. The NF-κB site at 5.8 kb is required for both basal and proinflammatory cytokines-induced promoter activity, whereas the NF-κB sites at 5.2, 5.5, and 6.1 kb exert a cooperative effect on cytokine-induced iNOS expression [Taylor et al., 1998]. In contrast to the distal NF-κB sites, previous work by Taylor et al. [1998] determined that neither deletion nor mutation of the proximal NF-κB site at −115 bp of the human iNOS promoter had a significant effect on cytokine-induced iNOS promoter activity, suggesting that this proximal NF-κB site is not essential for basal and proinflammatory cytokines-induced iNOS promoter activity.

By investigating the upstream kinases involved in NF-κB activation, we have obtained data suggesting that HNE blocks IKKα-enhanced IL-1β-induced NF-κB activation and iNOS expression. Interestingly, IKKα appears to be susceptible to inactivation by the formation of HNE/IKKα adducts. These results agree with a previous report showing that HNE specifically prevents LPS-induced phosphorylation of IκBα, whereas the profound effect of HNE on NF-κB is not due to interference

with the LPS-binding properties of the cells [Page et al., 1999]. The authors suggested that HNE inhibited IκBα phosphorylation at a signaling stage located downstream from the LPS receptor level. In an another study, Ji et al. [2001] reported higher susceptibility of IKKs to inactivation by HNE binding in Jurkat T-cells. The authors suggest that HNE inhibits IKKs activity by direct reaction with a cysteine residue. These results concur with the known chemical reactivity of HNE and available structural information on IKKs. The α,β-double bond of HNE reacts spontaneously, via Michael addition, to the sulfhydryl group of cysteine, the ε-amino group of lysine, and the imidazole function of histidine, but the sulfhydryl group shows the highest reactivity at neutral pH [Esterbauer et al., 1991]. Various cellular mechanisms responsible for IKKs inactivation have been proposed. These mechanisms include direct interaction of ROS (oxidation process) [Korn et al., 2001] or NO (S-nitrosylation process) [Reynaert et al., 2004] with cysteine residues present in the IKK complex. In addition, others proteins kinases family have been shown to be modulated by HNE binding. Parola et al. reported that c-Jun amino-terminal kinases (JNKs) nuclear translocation and activation are strictly related to the formation of HNE/JNK adducts [Parola et al., 1998].

In conclusion, we have studied molecular elements of the stress responses in OA chondrocytes by examining the role of the ATF/CRE and NF-κB signaling pathway in the regulation of COX-2 and iNOS. Our data indicate that ATF/CRE alone or possibly in combination with other coordinated signaling events is necessary for HNE-induced COX-2 production in OA chondrocytes. In addition, it was observed that HNE-induced COX-2 expression is a p38 MAPK activation dependent. However, treatment with HNE appears to block selectively the signaling events that are required for IκBα degradation and NF-κB activation. Inhibition of NF-κB-regulated iNOS expression by HNE may be attributed to IKKα inactivation by HNE/IKKα adducts formation. Our study has several important implications and suggests that HNE production in OA articular tissues contributes to inflammatory responses in OA by upregulation of COX-2 expression and by limiting the magnitude of transcriptional expression of iNOS.

ACKNOWLEDGMENTS

Dr. Benderdour is a research scholar of the FRSQ. We thank Drs M. Karen, R.J. Davis, and R.A. Shapiro for their respective generous gifts of the IKKα/β and p38 MAPK expression plasmids, and iNOS promoter constructs.

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