ARTHRITIS & RHEUMATISM
Vol. 50, No. 11, November 2004, pp 3549–3560
DOI 10.1002/art.20596

Effect of Hypoxia and Reoxygenation on Gene Expression and Response to Interleukin-1 in Cultured Articular Chondrocytes

G. Martin,[1] R. Andriamanalijaona,[1] S. Grässel,[2] R. Dreier,[3] M. Mathy-Hartert,[4] P. Bogdanowicz,[1] K. Boumédiene,[1] Y. Henrotin,[4] P. Bruckner,[3] and J.-P. Pujol[1]

Objective. **To determine the effects of hypoxia and reoxygenation on the metabolism of chondrocytes and their response to interleukin-1β (IL-1β). The study included activation of hypoxia-inducible factor 1 (HIF-1), NF-κB, and activator protein 1 (AP-1) transcription factors, expression of matrix components and metalloproteases and transforming growth factor β (TGFβ) and TGFβ receptors, and production of nitric oxide (NO) and prostaglandin E_2 (PGE_2).**

Methods. **Bovine articular chondrocytes (BACs) were cultured to confluency in either 5% O_2 (hypoxia) or 21% O_2 (normoxia) in media supplemented with 10% fetal calf serum (FCS). BACs were preincubated for 18 hours in media with 1% FCS only and then incubated for 24 hours in the presence of IL-1β. For reoxygenation experiments, cells were treated in the same way in 5% O_2, except that cultures were transferred to normal atmospheric conditions and used after 4 hours for RNA extraction or after 30 minutes for cytoplasmic or nuclear protein extraction.**

Results. **In hypoxic and reoxygenated chondrocytes, we observed strong DNA binding of HIF-1. IL-1β–induced DNA binding of NF-κB and AP-1 was significantly higher in hypoxic and reoxygenated cultures than in normoxia. Greater activation of the MAPKs was also observed with IL-1β treatment in hypoxia compared with normoxia. Steady-state levels of type II collagen and aggrecan core protein messenger RNA (mRNA) were decreased by IL-1β in all instances. Matrix metalloprotease 1 (MMP-1) and MMP-3 mRNA were increased by IL-1β in normoxia and hypoxia, whereas only MMP-3 mRNA was enhanced in reoxygenated cultures. The MMP-2 mRNA level was not significantly affected by IL-1β in normoxia or hypoxia, whereas it was enhanced in reoxygenated cultures. MMP-9 mRNA was dramatically decreased by IL-1β only in low oxygen tension. Tissue inhibitor of metalloproteinases 1 (TIMP-1) message was significantly enhanced by the cytokine in most instances, whereas TIMP-2 message was markedly decreased by IL-1β in reoxygenated cultures. Stimulation of TGFβ1 expression by IL-1β was observed only in normal atmospheric conditions. One of the more striking findings of the study was the greater stimulating effect of IL-1β on NO production observed in hypoxia, which was much higher than in normoxia, whereas the reverse was observed for IL-1β–induced PGE_2 production.**

Conclusion. **Oxygen level and reoxygenation stress significantly modulate gene expression and the response of articular chondrocytes to cytokines such as IL-1β. In hypoxic conditions, which mimic the in vivo condition of cartilage, the effects of IL-1β on both synthesis and degradative processes are significantly different from those in normoxia, conditions that are unlikely encountered by chondrocytes in a normal state. In low oxygen tension, high IL-1β–induced NO production is associated with a significant decrease in PGE_2 synthesis. These data should influence our concept of the role of oxygen in the pathophysiology of joint disease and may help define the best conditions in which to develop bioartificial cartilage.**

Studies to date on the role of hypoxia in the skeleton have involved tissues or cells of mesenchymal origin. Reports are conflicting as to whether chondro-

Supported by an unrestricted educational grant from UCB Pharma (Belgium) and by the Regional Council of Lower Normandy.

[1]G. Martin, PhD, R. Andriamanalijaona, PhD, P. Bogdanowicz, PhD, K. Boumédiene, PhD, J.-P. Pujol, PhD: Laboratory of Connective Tissue Biochemistry, Caen, France; [2]S. Grässel, PhD: Orthopedic University Clinic, Regensburg, Germany; [3]R. Dreier, PhD, P. Bruckner, PhD: Institute for Physiological Chemistry and Pathobiochemistry, Munster, Germany; [4]M. Mathy-Hartert, PhD, Y. Henrotin, PhD: University of Liege, Liege, France.

Address correspondence and reprint requests to J.-P. Pujol, PhD, Laboratory of Connective Tissue Biochemistry, Faculty of Medicine, 14032 Caen Cedex, France. E-mail: pujol@ibba.unicaen.fr.

Submitted for publication November 12, 2003; accepted in revised form July 28, 2004.

cytes in growth plate cartilage, which is normally avascular, are hypoxic (1,2). In the case of adult cartilage, however, there is evidence that the oxygen supply to resident chondrocytes is rather limited (3,4). Oxygen, as well as other nutrients, must diffuse into the tissue from the synovial fluid. Microelectrode studies have shown that an oxygen gradient exists in cartilage, with the superficial layers and deep zones having tension of ~6% O_2 and <1% O_2, respectively (4). The possibility also exists that changes in oxygen concentration may occur during joint diseases, such as rheumatoid arthritis (RA) and osteoarthritis (OA). For example, it has been suggested that in these diseases decreased capillary density of the synovium may contribute to reduced oxygen tension in the joint (5,6). In contrast, it is likely that in advanced fibrillated stages of OA cartilage, a higher oxygen supply can reach chondrocytes in altered zones of the tissue.

Articular cartilage is not the only tissue living in low oxygen tension. For example, measurements of bone marrow aspirates from human donors yielded mean P_{O_2} values of 6.4% (7,8). In inflamed tissue, infected tissue, tumors, wounds, and fracture sites, the P_{O_2} level could be considerably lower (9). In the case of chondrocytes, it has been suggested that the low oxygen tension prevailing in cartilage imposes energy limitations and regulates matrix synthesis (10). Nevertheless, chondrocytes have been shown to be well adapted to these conditions and capable of maintaining the energy charge of the cell, principally based on the glycolytic pathway (11).

Thus, conventional cell culture using atmospheric air exposes chondrocytes to oxygen tensions that are much higher than pathophysiologic levels (12). As a consequence, biochemical changes associated with variations in oxygen delivery are still poorly understood. Results of a few studies have indicated that articular chondrocytes cultured in low oxygen tension show down-regulation of type II collagen, no change in aggrecan messenger RNA (mRNA) levels, and up-regulation of tissue inhibitor of metalloproteinases 1 (TIMP-1) (13–16). The oxygen level may also modulate the chondrocyte response to the cytokines implicated in joint diseases, including interleukin-1 (IL-1), tumor necrosis factor α (TNFα), and transforming growth factor β (TGFβ). In this regard, it has been reported that hypoxia decreases IL-1α– and TNFα-induced nitric oxide (NO) production in porcine cartilage explants (17).

The pathogenesis of joint diseases is associated with cartilage breakdown due to the induction of several metalloproteases in both synoviocytes and chondrocytes, together with reduced synthesis of specific matrix components including type II collagen and aggrecan. The cytokines IL-1 and TNFα are key factors in this metabolic dysfunction (18,19). They induce expression of the NO synthase 2 (NOS2) (20–22) and cyclooxygenase 2 (COX-2) (23,24) enzymes, resulting in increased production of NO and prostaglandin E_2 (PGE_2). It has been shown that activation of NF-κB and activator protein 1 (AP-1) transcription factors by IL-1β is involved in the expression of several proinflammatory genes, including COX-2 (25), NOS2 (26–28), and matrix metalloproteases (MMPs) (29–31).

IL-1β is also capable of stimulating TGFβ1 expression in articular chondrocytes by a mechanism that is NO-dependent (Martin G, et al: unpublished observations). In turn, TGFβ1 can antagonize most of the deleterious effects of IL-1β and has been proposed as a factor promoting cartilage repair (32). It is likely that this interplaying network of cytokines/growth factors could be influenced by the oxygen tension present in the microenvironment of chondrocytes. Furthermore, degeneration of the articular cartilage in OA causes fibrillation in the tissue, and sometimes microfractures of the subchondral bone may also occur. These processes probably disrupt the gradient of oxygen in the cartilage and produce reoxygenation of the chondrocytes. The presence of higher oxygen tension may facilitate production of reactive oxygen species, which are known to play signaling roles (for review, see ref. 33) and could also contribute to the modulation of chondrocyte gene expression.

In the present study, we examined the effect of hypoxia (5% O_2) and reoxygenation to atmospheric air on several functional parameters of articular chondrocytes related to both anabolic and catabolic aspects of their activity. Cells were treated or not treated with IL-1β to determine whether oxygen tension could modulate the response of chondrocytes to this cytokine. Furthermore, the expression of TGFβs and their receptors was studied as a critical aspect of cartilage repair potential.

MATERIALS AND METHODS

Reagents. Mouse anti–phospho-MAPK and rabbit anti–MAPK-1/2 monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). P50, c-Rel, p65, c-Jun, c-Fos, and JunB polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit or anti-mouse horseradish peroxidase–labeled secondary antibodies (Santa Cruz Biotechnology) were used for detection with an ECL Plus kit (Amersham Pharmacia Biotech, Orsay, France). The oligonucleotide probes were supplied by Invitro-

gen (Cergy Pontoise, France) and were as follows: for HIF-1, 5′-TTGTGAGACGTGCGGCTTCCCA-3′; for AP-1, 5′-CGCTTGATGAGTCAGCCCGGAA-3′; for NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′. All other chemicals were of the highest purity (Sigma-Aldrich, St. Quentin Fallavier, France). Human recombinant IL-1β was a generous gift from Dr. Soichiro Sato, Shizuoka, Japan.

Culture and treatment of articular chondrocytes. Chondrocytes were isolated from metacarpophalangeal joints of freshly slaughtered calves by enzymatic treatment (12). Slices of cartilage were then digested in a special plastic chamber at low oxygen tension in order to prevent any reoxygenation of the chondrocytes during the isolation process (see below). Sequential digestion with type XIV protease and type I collagenase was performed, as previously described (12), in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) previously equilibrated at 5% O_2. The cells were seeded in DMEM containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and antibiotic/antimycotic (penicillin, erythromycin, and Fungizone). Three samples were cultured: two at 5% O_2 (hypoxia and reoxygenation) and one at 21% O_2 (normoxia). A sealed chamber (model 818-GBB/22; Bioblock Scientific, Illkirch, France), equipped with gloves, was used. A small culture incubator was placed inside, and vessels were introduced by a special airlock. The chamber was previously equilibrated with 5% O_2 by flushing a gas mixture (N_2 + CO_2) without oxygen (Bactal 2 Pro; Air Liquide, Mitry Mory, France). The oxygen tension was monitored with a specific probe (model 74223; Bioblock Scientific). The oxygen concentration of the culture media was reduced from 20% to 5% O_2 by bubbling a gas mixture without oxygen (1.5 hours for a 500-ml flask). The oxygen level was measured by inserting a probe into the culture media (Oxi 330/set; Wissenschaftlich-Techniche Werkstätten, Weilheim, Germany). Phenol red, generally used as a pH indicator, was absent in the medium because of its antioxidant properties.

To induce reoxygenation, some cell cultures were transferred from hypoxic to atmospheric conditions and used after 30 minutes for electrophoretic mobility shift assay (EMSA) analysis and after 4 hours for Northern blotting experiments. For the reoxygenation step, culture media were also replaced with fresh media containing 21% O_2.

Preparation of cytoplasmic and nuclear extracts. Cells were rinsed twice with phosphate buffered saline (PBS) and scraped in a hypotonic buffer (34). After centrifugation at 10,000*g* for 10 minutes, the resulting supernatants were used as cytoplasmic extracts. The pellet was incubated in hypertonic buffer for 4 hours at 4°C and centrifuged at 10,000*g* for 30 minutes to provide nuclear extracts (34). The protein amount was determined by Bradford's colorimetric procedure (Bio-Rad SA, Ivry sur Seine, France).

Western blot analysis. Cellular lysates (15 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (35) and electrophoretically transferred to polyvinylidene difluoride membranes (NEN Life Science Products, Zawentem, Belgium). The membranes were then treated as previously described (36).

EMSA. Nuclear extracts (10 μg protein) were incubated in binding buffer for 30 minutes at 25°C with complementary DNA (cDNA) probes, previously labeled with [γ-^{32}P]ATP (25 fmoles) using T4 polynucleotide kinase (Life Technologies). The final binding reaction for NF-κB was performed in 20 m*M* HEPES (pH 7.5), 50 m*M* KCl, 4 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 0.05% Nonidet P40, 20% glycerol, 1 mg/ml bovine serum albumin, and 0.025 m*M* poly(dI-dC). The binding reaction mixture for HIF-1 and AP-1 was composed of 20 m*M* HEPES, 2 m*M* Tris HCl (pH 8), 1 m*M* DTT, 80 m*M* NaCl, 10% glycerol (volume/volume), 0.2 m*M* phenylmethylsulfonyl fluoride, 0.4 m*M* EDTA, and 0.2 m*M* EGTA. The samples were then submitted to 8% PAGE in 0.5× TBE (45 m*M* Tris [pH 7.8], 45 m*M* boric acid, and 1 m*M* EDTA) and visualized by autoradiography (37).

Determination of TGFβ1 and TGFβ2 proteins by enzyme-linked immunosorbent assay (ELISA). TGFβ1 and TGFβ2 proteins were assayed in culture media, using an ELISA kit (R&D Systems, Minneapolis, MN).

RNA extraction and Northern blot analysis. *Preparation of probes.* Probes were obtained by reverse transcription–polymerase chain reaction (RT-PCR) of total RNA extracted from 5×10^6 chondrocytes, using the RNeasy Mini protocol (Qiagen, Courtaboeuf, France) or the guanidium isothiocyanate phenol chloroform procedure (38). Samples were treated with DNase during the RNA isolation procedure according to the manufacturer's protocol (Invitrogen). The RT reaction and PCR amplification were performed as previously described (39). Cycling parameters included an initial denaturation step for 5 minutes at 95°C, followed by 35 or 40 cycles of 30 seconds at 95°C, 1 minute at various annealing temperatures according to the temperature of the primers (55°C for A–C; 60°C for D, E, H, I, and J; 56.5°C for F and G), and 1 minute at 72°C.

For quantification of MMP and TIMP mRNAs, PCR products were purified using a PCR purification kit (Qiagen), subcloned into the pCR II-TOPO vector (Invitrogen) and subsequently sequenced for identification (ABI Prism genetic analyzer, model 310; Amersham Pharmacia Biotech). The GAPDH probe was a gift from Dr. H. Kresse's group (Munster, Germany).

Primer sequences. The primer sequences were as follows: for type II collagen (648 bp), sense 5′-GACCCCATGCAGTACATG-3′, antisense 5′-GACCGTCTTGCCCCACTT-3′; for aggrecan core protein (200 bp), sense 5′-CCCTGGACTTTGACAGGGC-3′, antisense 5′-AGGAAACTCGTCCTTGTCTCC-3′; for 18S RNA (100 bp), sense 5′-CTTTTCAGAGGGACAAGTGG-3′, antisense 5′-CCTACGGAAACCTTGTTACG-3′; for MMP-1 (442 bp), sense 5′-GGGAAATCCTGTTGGGAGAACA-3′, antisense 5′-GATGGCCTGGATCCCATCAA-3′; for MMP-2 (334 bp), sense 5′-CGCTCGTGCCTTCCAAGTCT-3′, antisense 5′-TGGTGGAACACCAAAGGAAGC-3′; for MMP-3 (429 bp), sense 5′-TGGCTCATGCCTACCCACCT-3′, antisense 5′-AAGAGATCAAATGAAATTCAGGTTC-3′; for membrane type 1 (MT1)–MMP (625 bp), sense 5′-TCAAGGCCAATGTTCGAAGGA-3′, antisense 5′-GGCCACGGTGTCAAAGTTCC-3′; for TIMP-1 (469 bp), sense 5′-GCCTCTGGCATCCTGTTGCT-3′, antisense 5′-GGTCCGTCCACAAGCAGTGA-3′; for TIMP-2 (500 bp), sense 5′-GATCAGGGCCAAAGCAGTCAA-3′, antisense 5′- CGATGTCCAGAAACTCCTGCTTG-3′; for MMP-9 (495 bp), sense 5′- GAACCACGAACCAACCTCAC-3′, antisense 5′-CATCTCCCTGAATGCCTTG-3′.

Labeling of the probes. Type II collagen, aggrecan, and 18S probes were ^{32}P-radiolabeled, using a random priming kit (Invitrogen). The other probes were labeled with the Prime-A-Gene kit (Promega, Charbonnières, France) according to the manufacturer's protocol.

Northern blotting. Type II collagen and aggrecan Northern blots were prehybridized (1 hour at 42°C) and hybridized (18 hours at 48–60°C) in Na_2HPO_4 (pH 7.2–7.4)/SDS 20% (2:1 [v/v]). The blots were washed several times in 2× standard saline citrate (SSC) and 0.1% SDS at 42°C. Final washes were in 0.1× SSC plus 0.1% SDS at 42°C. The ^{32}P-radiolabeled cDNA–mRNA hybrids were visualized by autoradiography. The signals were then measured by densitometric scans of the x-ray film (Kodak X-Omat; Kodak, Rochester, NY) and quantified by the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

For the other blots, filters were hybridized either overnight (MMPs and TIMPs) or for 1 hour (GAPDH) at 60°C, after prehybridization with 5 ml of QuikHyb hybridization solution (Stratagene, Europe, Amsterdam, The Netherlands) for 1 hour at 60°C. Filters were subsequently washed, once or twice each, in washing buffer containing 2× SSC plus 0.1% SDS, 1× SSC plus 0.1% SDS, 0.2× SSC plus 0.1% SDS, and 0.1× SSC plus 0.1% SDS for each, 10 minutes at 60°C. Filters were exposed to Storm phosphoimager screens (Amersham Pharmacia Biotech) either overnight (MMPs and TIMPs) or for 4–5 hours (GAPDH), scanned with a Storm phosphoimager (Amersham Pharmacia Biotech), and subjected to a densitometric evaluation with ImageQuant analysis software (Amersham Pharmacia Biotech).

Filters were repeatedly hybridized with up to 5 different probes and between hybridizations, filters were stripped with 10 m*M* EDTA, 10 m*M* Tris, and 0.1% SDS for 10 minutes at 85–95°C. Subsequently, filters were equilibrated twice in 2× SSC at room temperature.

Semiquantitative RT-PCR. Three micrograms of RNA was transcribed in a 50-μl volume containing 10 μl of 5× First Strand buffer, 20 μM oligo(dT)$_{16}$, 1 μl of recombinant ribonuclease inhibitor (RNAseOUT; Invitrogen) (40 units/μl), 3 μl of dNTPs (10 m*M* each), and 1 μl of Moloney murine leukemia virus (200 units/μl) for 15 minutes at 42°C and 5 minutes at 99°C. PCR was performed with 3 μl of cDNA in 40 μl of PCR reaction sample with 0.2 μl of *Taq* polymerase (15 units/μl), 1 μl of dNTPs (10 m*M* each), 1 μl of 50 m*M* $MgCl_2$ and 20 p*M* of each primer. Amplification of the cDNA sequence was performed by PCR, using the following primer sequences: for TGFβ1, sense GCCCTGGACACCAACTATTGC, antisense GCTGCACTTGCAGGAGCGCAC; for TGFβ2, sense GCTTTGGATGCGGCCTATTGC, antisense GCTGCATTTGCAAGACTTTAC; for TGFβ receptor type I (TGFβRI), sense ATTGCTGGACCAGTGTGCTTCGTC, antisense TAAGTCTGCAATACAGCAAGTTCCATTCTT; for TGFβRII, sense CGCTTTGCTGAGGTCTATAAGGCC, antisense GATATTGGAGCTCTTGAGGTCCCT. The following amplification protocol was used: 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C. PCR products were analyzed in 2% agarose gels stained with ethidium bromide. The band densities were quantified using ImageQuant. The relative expression amount of messengers was calculated as the ratio to the expression of 18S mRNA, as a housekeeping gene reference. The procedure was repeated in 3 different experiments.

Measurement of NO and PGE_2 production. NO production was assessed by measuring the concentration of nitrate/nitrite in media, using the Griess reaction (40). PGE_2 was assayed in the culture media according to a previously described radioimmunoassay (41).

Gelatin zymography. Fifty-microliter aliquots of medium were mixed with equal volumes of 2-fold concentrated sample loading buffer (2 m*M* EDTA, 2% SDS, 20% glycerol, 0.02% bromphenol blue, 20 m*M* Tris HCl [pH 8.0]) and subjected to electrophoresis on a 1% gelatin–containing 4.5–15% gradient SDS polyacrylamide gel. The gels were washed twice for 30 minutes in 2.5% Triton X-100, rinsed in distilled water, and developed for 16 hours at 37°C in 50 m*M* Tris HCl (pH 8.5) containing 5 m*M* $CaCl_2$. Finally, they were stained with Coomassie brilliant blue R250 (Serva, Heidelberg, Germany) to visualize protease activity and photographed.

Statistical analysis. Three different experiments were performed. Data are presented as the mean ± SEM. *P* values were calculated by Student's *t*-test.

RESULTS

Strong HIF-1 DNA binding induced by hypoxia and reoxygenation. We first examined the binding activity of the redox-sensitive transcription factor HIF-1, which is known to regulate the expression of glycolytic enzymes in most cell types. In normoxia, HIF-1 binding activity was very low or undetectable, but it strongly increased in hypoxia (Figure 1A). When cells were reoxygenated after culturing in low oxygen tension, HIF-1 binding was maintained at the same level. Exposure to IL-1β did not induce a significant change in HIF-1 binding activity in both situations. Competition analysis, using a wild-type probe, revealed the specificity of this complex (Figure 1A).

Enhancement of IL-1–induced NF-κB and AP-1 DNA binding by hypoxia and reoxygenation. To investigate the influence of normoxia, hypoxia, and reoxygenation on the response of articular chondrocytes, we performed a series of EMSA analyses, using consensus DNA binding sequences for NF-κB and AP-1 transcription factors. Nuclear extracts were obtained from BACs cultured in either normoxia (21% O_2) or hypoxia (5% O_2) and treated with IL-1β (10 ng/ml) for several time periods. Part of the cells cultured in hypoxia were submitted to a 30-minute period of reoxygenation to produce an oxidative stress. As expected, the binding activities of NF-κB and AP-1 complexes were strongly induced by the exposure of chondrocytes to IL-1β in normoxia (Figures 1B and C). One-hour treatment with IL-1β was found to be sufficient to produce the binding of 3 NF-κB complexes (Figure 1B), whereas 6-hour treatment was required to clearly stimulate the binding of AP-1 (Figure 2A). IL-1β–induced binding of NF-κB

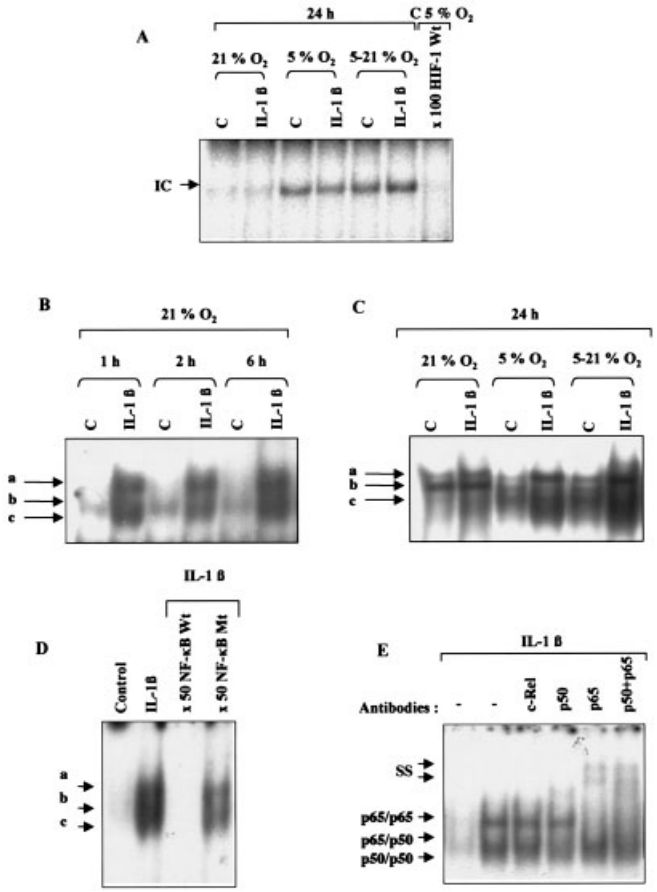

Figure 1. Hypoxia-inducible factor 1 (HIF-1) and NF-κB DNA binding activity under interleukin-1β (IL-1β) treatment: effect of oxygen tension and reoxygenation. Bovine articular chondrocytes were cultured in normoxia (21% O_2) or hypoxia (5% O_2) and were treated or not treated with IL-1β (10 ng/ml) for several time periods (1, 2, 6, and 24 hours). Part of the cells cultured in 5% O_2 were transferred from hypoxic to atmospheric conditions in order to induce an oxidative stress and used after 30 minutes. At this stage, nuclear extracts were prepared and used in electrophoretic mobility shift assay. The binding of HIF-1 was studied using a specific cDNA probe. **A,** The binding specificity of complexes induced by hypoxia was assessed with unlabeled HIF-1 wild-type (Wt) duplexes as competitors. **B** and **C,** The binding of NF-κB was studied using a specific cDNA probe. **D,** The binding specificity of complexes induced by IL-1β was characterized using unlabeled NF-κB wild-type or mutant (Mt) duplexes as competitors. **E,** Inducible complexes were identified using anti–c-Rel, anti-p50, and anti-p65 antibodies. C = control; IC = inducible complex; SS = supershift. a, b, and c denote binding complexes. Results shown are representative of 3 independent experiments.

and AP-1 was greater in hypoxia compared with normoxia (Figures 1C and 2B). Moreover, hypoxic conditions were found to increase the basal binding activity of AP-1. Interestingly, it must be noted that an additional NF-κB protein complex, with lower molecular weight, appeared in IL-1–treated hypoxic cultures compared with those treated in normoxic conditions (Figure 1C).

Competition analysis, using wild-type or mutated probes, revealed the specificity of these complexes (Fig-

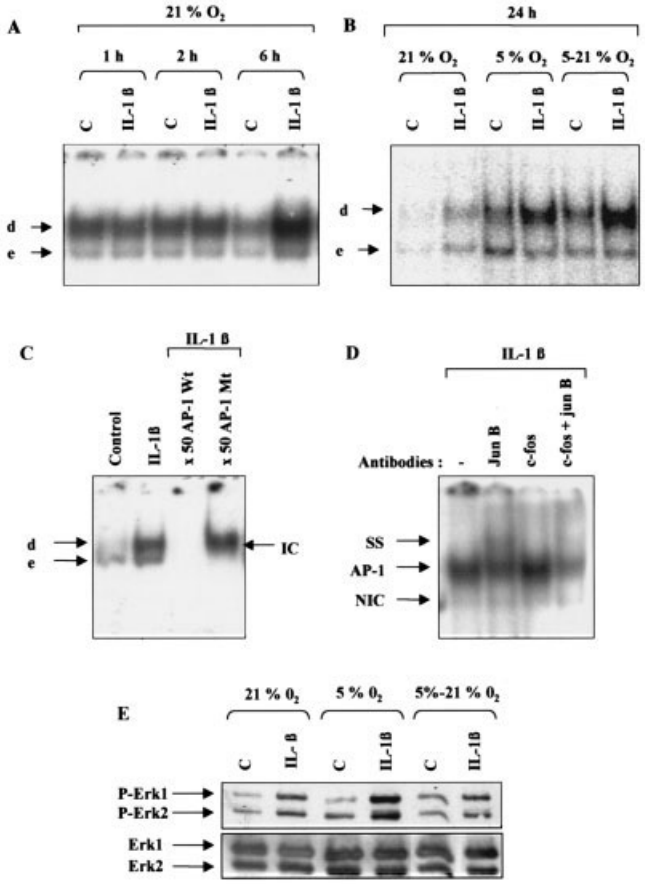

Figure 2. Effect of oxygen tension and reoxygenation on activator protein 1 (AP-1) DNA binding and ERK1/2 MAPK activity under interleukin-1β (IL-1β) treatment. Bovine articular chondrocytes (BACs) were cultured as described in Figure 1, and nuclear extracts were used in electrophoretic mobility shift assay (**A** and **B**). The binding specificity of complexes induced by IL-1β was characterized using unlabeled NF-κB duplexes, wild-type (Wt), or mutant (Mt) duplexes as competitors (**C**). Finally, inducible complexes were identified using anti–c-fos and anti-JunB antibodies (**D**). BACs were cultured either in normoxia (21% O_2) or hypoxia (5% O_2) and treated with IL-1β (10 ng/ml) for 30 minutes. Part of the cells cultured in hypoxia were transferred to a normoxic environment for 15 minutes before the end of IL-1β treatment in order to generate an oxidative stress. Cytoplasmic extracts were prepared and used for Western blot analysis, using anti–phospho-ERK1/2 (P-Erk1 and P-Erk2, respectively) (**E**). The protein–antibody complexes were visualized by chemiluminescence, using horseradish peroxidase–conjugated secondary antibody. Results shown are representative of 3 independent experiments. C = control; IC = inducible complex; SS = supershift; NIC = noninducible complex. d and e denote binding complexes.

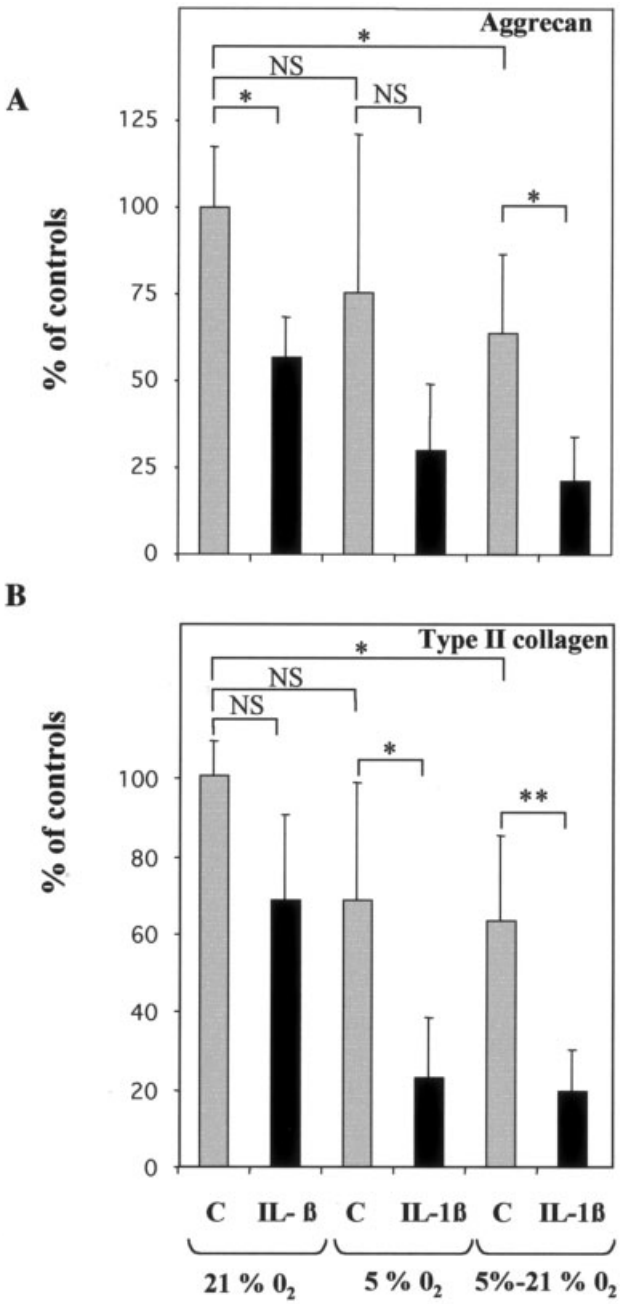

Figure 3. Modulation of the effect of interleukin-1β (IL-1β) on aggrecan core protein and type II collagen expression by oxygen tension and oxidative stress. Bovine articular chondrocytes were cultured as described in Figure 1 and treated with IL-1β (10 ng/ml) for 24 hours. Part of the cells cultured in 5% O_2 were submitted to 4-hour reoxygenation. Total RNA was isolated and used in Northern blotting analysis, with 2 labeled probes corresponding to aggrecan core protein **(A)** and COL2A1 **(B)** cDNA. Hybridization with a probe corresponding to 18S RNA is shown as an internal control of RNA loading. Histograms show the mean and SEM values obtained by densitometric scanning of membranes prepared with RNA from 3 different experiments. The normoxic control (C) was arbitrarily set at 100%. NS = not significant. $* = P < 0.05$; $** = P < 0.01$.

ures 1D and 2C). In an attempt to identify the NF-κB components induced by IL-1β, we performed supershift assays with specific antibodies to NF-κB subunits. Antibodies to p65 and to p50 subunits caused a disappearance of the complex, suggesting that IL-1β induced the binding of a p65 homodimer and a heterodimer that could be formed by association of p65 and p50 subunits (Figure 1E). A similar approach demonstrated that IL-1β specifically activated the c-Fos and JunB subunits of AP-1 (Figure 2D). Reoxygenation of hypoxic cultures was found to increase the IL-1β–induced binding of NF-κB and AP-1, as compared with cultures maintained in hypoxia.

Greater IL-1β stimulation of MAPKs in low oxygen tension. To further study the effect of oxygen tension on the signaling pathways upstream of NF-κB and AP-1 DNA binding, we performed Western blot analysis of MAPKs. A 30-minute treatment with IL-1β induced phosphorylation of ERK-1 and ERK-2 in the chondrocytes, reflecting activation of the kinases (Figure 2E). In addition, the bands were always of greater intensity for chondrocytes cultured in low oxygen tension, indicating that IL-1β stimulation was more effective in hypoxia than in normoxia. In contrast, reoxygenation of hypoxic cultures caused a decrease of IL-1β effect, as compared with hypoxic controls.

Modulation of IL-1β effect on aggrecan core protein and type II collagen mRNA steady-state levels by oxygen tension. Northern blot analysis revealed that the mRNA levels of both aggrecan core protein and type II collagen were lower in hypoxic and reoxygenation conditions, as compared with normoxia (Figure 3A and B). However, the decrease was not statistically significant between normoxia and hypoxia. As expected, IL-1β treatment (24 hours) caused a reduction of these mRNA amounts in the 3 experimental conditions. This downregulation was more marked for collagen in hypoxia and reoxygenation conditions than in normoxia (Figure 3B).

Influence of oxygen tension on IL-1β regulation of MMP expression. As studied by Northern blotting, the response of MMP genes to oxygen tension and IL-1β was dependent on the MMP type. As expected, MMP-1 and MMP-3 mRNAs were increased by treatment of chondrocytes with IL-1β in both normoxic and hypoxic conditions, although the difference was not statistically significant (Figures 4A and C). However, in reoxygenated cultures, only MMP-3 expression was enhanced by IL-1β, with no change of the MMP-1 mRNA level observed in that situation. MMP-2 and MMP-9 genes were found to respond in a manner different from that of MMP-1 and MMP-3. In normoxic cultures of chondrocytes, MMP-2 and MMP-9 mRNA levels were not significantly affected by IL-1β (Figures 4B and D). In

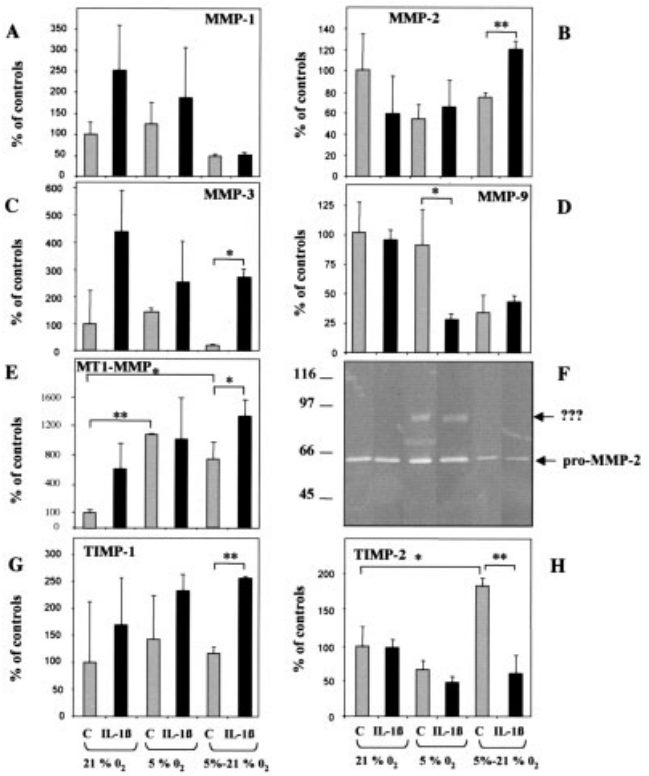

Figure 4. Effect of hypoxia and reoxygenation on matrix metalloprotease (MMP) and tissue inhibitor of metalloproteinases (TIMP) expression by chondrocytes treated or not treated with interleukin-1β (IL-1β). Total RNA from chondrocyte cultures treated as described in Figure 1 was used in Northern blot analysis to quantify the mRNA steady-state levels of MMP-1 **(A),** MMP-2 **(B),** MMP-3 **(C),** MMP-9 **(D),** membrane type 1 (MT1)–MMP **(E),** TIMP-1 **(G),** and TIMP-2 **(H)** genes. Data are the mean and SEM of 3 experiments. The gelatinolytic activities produced by chondrocyte cultures was analyzed by zymography **(F)**. Culture media from cultures treated as described in Figure 1 were subjected to electrophoresis on a 1% gelatin–containing 4.5–15% gradient sodium dodecyl sulfate–polyacrylamide gel (see Materials and Methods). Protease activity was visualized with Coomassie brilliant blue R250 staining. The apparent lower amount in reoxygenation samples is attributable to the fact that the culture medium was washed out and replaced with fresh fully oxygenated medium to ascertain rapid reoxygenation. As a consequence, the values are likely to represent production over the last 4 hours. C = control. $* = P < 0.05$; $** = P < 0.01$. ??? = unidentified molecule.

low oxygen tension, MMP-2 mRNA was reduced by half as compared with normoxic conditions, but IL-1β did not affect this level (Figure 4B). In contrast, MMP-9 mRNA was significantly decreased by IL-1β in hypoxia (Figure 4D). Finally, in reoxygenated cultures, the MMP-2 mRNA level was enhanced by IL-1β, while MMP-9 mRNA was not affected. The expression of a membrane-type metalloprotease, MT1-MMP, was also investigated. First, we observed that the mRNA steady-state level of this gene was dramatically increased in hypoxia and in reoxygenated cultures, as compared with normal atmosphere (Figure 4E). IL-1β treatment caused an increase of MT1-MMP mRNA in normoxia and reoxygenation conditions, whereas no effect was observed in the setting of low oxygen tension.

In order to obtain more information on the enzymatic activity produced by chondrocytes in these experimental conditions, we also performed gelatin zymography analysis, using the conditioned culture media. We observed a band corresponding to a protein with an apparent molecular mass >66 kd in all of the samples, regardless of the treatment. The enzyme presumably causing this band is proMMP-2, because it comigrated with a gelatinolytic protein in media of human OA chondrocytes known to contain latent and active MMP-2. Supporting this hypothesis, an immunoblotting experiment with an antibody to MMP-2 (catalog no. IM33T; Oncogene Research Products, Cambridge, MA) revealed a band <66 kd under reduced conditions (results not shown). Culture at 5% O_2 led to production of an additional gelatinolytic molecule with an electrophoretic mobility slightly less than 97 kd. This proteolytic activity was not detectable in conditioned media of chondrocytes cultured at 21% O_2 or reoxygenated after a hypoxic period. It was not related to proMMP-9 or active MMP-9, because no reactivity was detected in immunoblots with an antibody to MMP-9 (Biomol, Plymouth Meeting, PA; catalog no. SA-106) (data not shown).

Effect of oxygen tension and IL-1β on TIMP-1 and TIMP-2 expression. Because TIMPs are key elements in the regulation of MMP activity in articular cartilage, we wanted to determine whether oxygen tension could modulate this expression. Northern blot analysis revealed that the variations of TIMP-1 and TIMP-2 mRNA in our experimental conditions were clearly different. The amount of TIMP-1 mRNA was not significantly altered by oxygen tension (Figure 4G), whereas it was increased by IL-1β, especially in reoxygenated cultures ($P < 0.01$). In contrast, the basal level of TIMP-2 mRNA was significantly augmented in reoxygenated cultures and dramatically reduced by IL-1β in these conditions (Figure 4H).

Effect of oxygen tension and IL-1β on the expression of TGFβ1, TGFβ2, and TGFβRs. TGFβs are implicated in cartilage homeostasis, exerting opposite effects against IL-1 on extracellular matrix metabolism. Therefore, it was interesting to study the expression of TGFβ1 and TGFβ2, together with that of TGFβRI and TGFβRII, in our system. Real-time PCR analysis was performed on total RNA, and ELISA was carried out to

assay TGFβ1 and TGFβ2 proteins in culture media. In low oxygen tension and after reoxygenation, the chondrocytes expressed more TGFβ1 mRNA and protein than in normal atmosphere (Figures 5A and C). This expression was significantly increased by IL-1β, at both the mRNA and protein levels ($P < 0.01$) in normoxia, whereas it was rather reduced in low oxygen tension and reoxygenation conditions. The production of TGFβ2 protein was found to be much lower in hypoxic conditions and in reoxygenated cultures than in normoxia

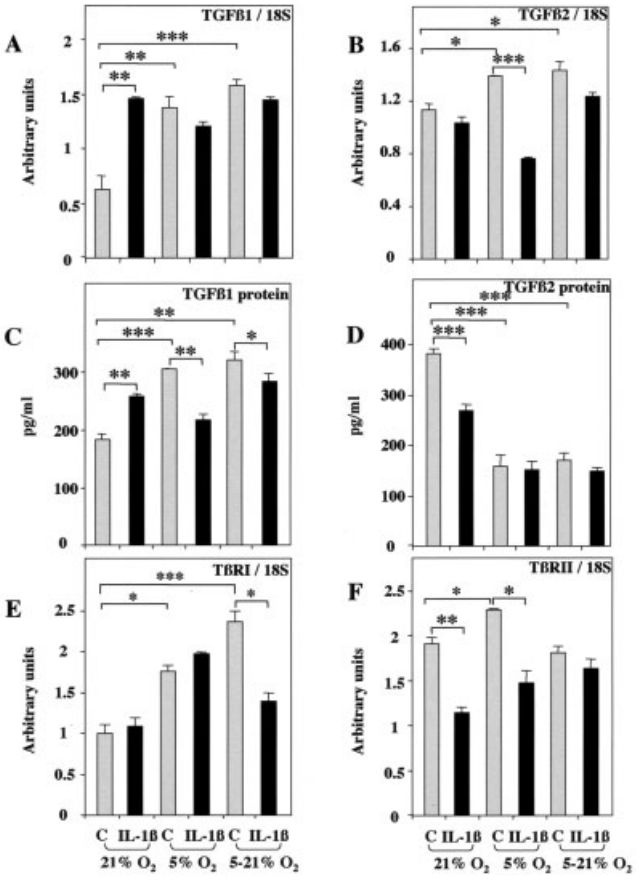

Figure 5. Effect of hypoxia and reoxygenation on transforming growth factor β1 (TGFβ1), TGFβ2, and TGFβ receptor (TβR) mRNA expression by chondrocytes treated or not treated with interleukin-1β (IL-1β). **A, B, E,** and **F,** Total RNA from chondrocytes treated as described in Figure 1 was used in reverse transcription–polymerase chain reaction analysis to quantify the mRNA steady-state levels of TGFβ1, TGFβ2, TβRI, and TβRII genes (see Materials and Methods). **C** and **D,** Media were collected to assay the levels of corresponding TGFβ1 and TGFβ2 proteins, using an enzyme-linked immunosorbent assay kit. Data are the mean and SEM of 3 experiments. The statistical significance of the differences between control (C) and IL-1β–treated samples was evaluated by Student's *t*-test. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$.

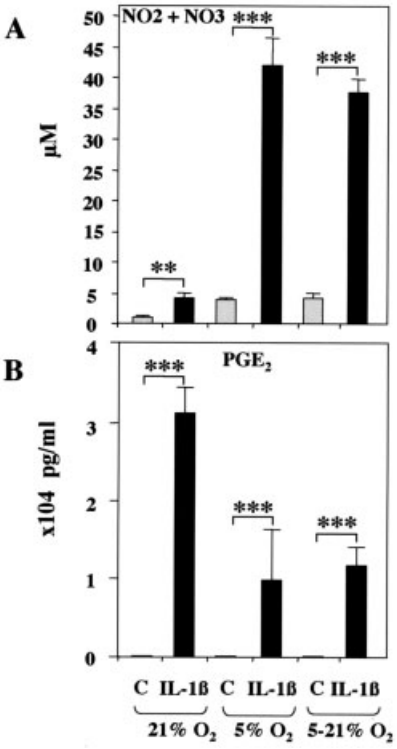

Figure 6. Production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) by normoxic, hypoxic, and reoxygenated chondrocyte cultures. Media from cultures treated as described in Figure 1 were collected and used to determine NO production by the Griess reaction **(A)** and PGE_2 levels by radioimmunoassay **(B).** The statistical significance of differences between control (C) and interleukin-1β (IL-1β)–treated cultures was evaluated by Student's *t*-test. Data are the mean and SEM. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$.

(Figure 5D). However, there was no correlation with the mRNA steady-state levels (Figure 5B), in contrast with TGFβ1 mRNA. In normoxic conditions, IL-1β treatment induced a decrease in TGFβ2 protein ($P < 0.001$), which was not reflected at the mRNA level (Figures 5B and D). The cytokine did not affect the protein level in the hypoxia and reoxygenation protocols, whereas the mRNA levels were decreased significantly in hypoxia (Figures 5B and D).

The mRNA level of TGFβRI was greater in chondrocytes cultured in low oxygen or reoxygenated after a hypoxic period than in normal atmosphere (Figure 5E). IL-1β did not affect this level in normoxia and hypoxia but caused a significant decrease in reoxygenated cultures. Contrasting with the TGFβRI mRNA level, the TGFβRII mRNA level did not significantly change, regardless of the conditions (Figure 5F). However, IL-1β reduced by ~2-fold the value of TGFβRII

mRNA in normoxia ($P < 0.01$) and by one-third the value in low oxygen tension ($P < 0.05$).

Differential effect of oxygen tension on IL-1β–induced NO and PGE_2 production. IL-1β–induced NO production by chondrocytes was significantly greater (8-fold) in hypoxia, compared with that in normal atmosphere (Figure 6A). Similar values were also observed for cultures that were reoxygenated after the low-oxygen period. Interestingly, the production of PGE_2 displays an opposite profile, being 3-fold lower in the setting of low oxygen and after reoxygenation of cells (Figure 6B). The data clearly indicate that inducible NOS (iNOS) and COX are differently regulated by oxygen tension.

DISCUSSION

Chondrocytes can survive in oxygen tension as low as <0.1% (14), indicating that these cells are well adapted to hypoxia (11). As a consequence, they display an unusual response to changes in oxygen tension. For example, in contrast to several cell types that increase their glycolytic enzyme expression and their anaerobic respiration (42), articular chondrocytes produce less lactic acid in low oxygen tension and are less metabolically active than in normoxia (11). Despite this existing knowledge, the response of articular chondrocytes to cytokines and growth factors in hypoxic conditions is still poorly understood.

Here, we found significantly greater HIF-1 DNA binding in hypoxic and reoxygenated chondrocyte cultures, compared with normoxic controls. These findings are consistent with those of a recent study showing that targeted deletion of HIF-1 in growth-plate chondrocytes renders them unable to maintain ATP levels and induces reduced synthesis of aggrecan and type II collagen mRNA in a hypoxic microenvironment (43). Taken together, these results clearly indicate the critical role of HIF-1 in the maintenance of anaerobic glycolysis and extracellular matrix synthesis of chondrocytes submitted to low oxygen tension.

Interestingly, our results revealed that the IL-1β–induced DNA binding of NF-κB and AP-1 was significantly enhanced in hypoxic and reoxygenated chondrocyte cultures. Furthermore, the NF-κB complexes formed in conditions of low oxygen tension and during reoxygenation appeared slightly different from those observed in normoxic control cultures. The intensity of the chondrocyte response to IL-1β is therefore greater in an environment of low oxygen tension, such as that found in living cartilage, suggesting that previous data obtained in normoxic conditions may have been underestimated. This can be extended to MAPKs, which were also activated by IL-1β to a greater extent in 5% O_2 concentration, a finding that confirms results of a previous study on human cartilage explants (44). In our study, we confirmed that type II collagen and aggrecan core protein mRNA levels were reduced in hypoxia (14,15), and we found that the IL-1β inhibitory effect on type II collagen expression was somewhat greater in hypoxia than in normoxia. The IL-1β–induced NF-κB activation can provide an explanation for the down-regulation of type II collagen expression, because it has recently been demonstrated that inhibition of COL2A1 transcription by IL-1β was mediated by the −57/+125-bp promoter region and was associated with induction of Egr-1, NF-κB, and ESE-1 binding activities in nuclear extracts (45).

A link can be established between IL-1 stimulation of MAPKs, AP-1, and NF-κB transcription factors, and the expression of several genes bearing AP-1– and NF-κB–responsive elements in their promoters, including MMPs, iNOS, and COX. For instance, it has previously been shown that IL-1 activation of MAPKs, NF-κB, and AP-1 was associated with up-regulation of some MMP gene expression, including MMP-3 and MMP-13, in BACs (46). The promoters of several MMPs are known to contain AP-1–responsive elements, and the role of AP-1 in their transcription has been largely documented (47,48). Implication of NF-κB, whose sites are not systematically found in all MMP promoters, has also been reported. For example, NF-κB activation is involved in the regulation of MMP-1 and MMP-13 genes in chondrosarcoma cells (49). In the present study, no systematic relationship between IL-1β–induced activation of AP-1 and NF-κB and the transcriptional activity of the MMP genes could be found, because the response to cytokine and oxygen tension was largely dependent on the MMP type.

Collagenase 1 (MMP-1) and stromelysin (MMP-3) are known to be expressed in a generally coordinated manner. Their promoters contain common regulatory sequences. In the present study, MMP-1 and MMP-3 mRNA levels were similarly increased by IL-1β in hypoxia and normoxia. However, we did not observe greater stimulation of these genes, despite the higher AP-1 and NF-κB binding in low oxygen tension. It is likely that other factors may contribute to regulate MMP-1 and MMP-3 gene transcription in hypoxic conditions. It is tempting to speculate that production of reactive oxygen species, which is likely to be greater in normoxia than in hypoxia, may synergize with IL-1, whereas the higher level of NO elicited by IL-1 in

hypoxia could reduce the cytokine effect on MMP-1 and MMP-3 (33). Interestingly, we observed that the MMP-3 mRNA level was significantly increased by IL-1β in reoxygenated cultures, while the amount of MMP-1 was not altered, indicating that there may be a differential regulation of these 2 MMP genes during oxidative stress.

Gelatinase A (MMP-2) and gelatinase B (MMP-9) have the same substrates, but they are controlled by different mechanisms in most cell types. Analysis of the 5′-flanking region of the MMP-2 gene has revealed striking differences with several MMP promoters, including the absence of TATA box and TRE sequence. Furthermore, the presence of a binding site for AP-2, several Sp1-binding elements, and 2 silencer sequences in the MMP-2 promoter suggests that MMP-2 could be regulated by tissue-specific repressors rather than activators. In the present study, these 2 MMPs were not stimulated by IL-1β in either normoxic or hypoxic cultures, in contrast to MMP-1 and MMP-3. Moreover, MMP-9 mRNA levels were strongly decreased by IL-1β in hypoxia. Interestingly, IL-1β was capable of stimulating MMP-2 expression during the oxidative stress induced by reoxygenation, suggesting that the reactive oxygen species produced in these conditions may up-regulate this MMP.

MT1-MMP is a membrane-associated MMP found in human cartilage (50). It possesses properties of collagenase and is able to activate the gelatinase MMP-2 and collagenase 3 (MMP-13) (51). Here, baseline expression of MT1-MMP mRNA was significantly increased in chondrocytes cultured in low oxygen tension (~12-fold) or reoxygenated (10-fold), compared with normoxic controls, suggesting that this gene could be HIF-1 dependent. Interestingly, MT1-MMP mRNA levels were further increased by exposure to IL-1β in reoxygenated cultures.

This study is the first to show that a gelatinolytic activity, which remains to be characterized, is expressed by chondrocytes in low oxygen tension. This activity was not apparently regulated by IL-1. Here, too, it is tempting to speculate that the expression of this enzyme could be linked to HIF-1 activation, as it is for MT1-MMP. TIMPs can control the biologic activity of MMP. TIMP-1 can bind activated forms of MMP-1 and MMP-3 and both latent and active MMP-9. In contrast, TIMP-2 is mainly associated with MMP-2. In agreement with this apparent functional difference, the promoters of these TIMP genes show variation in their regulatory elements: they both contain several Sp1 sites and 1 TRE sequence, but the TRE site of TIMP-2 is part of an inhibitory element. Thus, IL-1, TNFα, and TPA generally up-regulate TIMP-1, as well as MMP-1 and MMP-3, whereas TIMP-2 and MMP-2 are weakly influenced by these factors. This was the case in our study, because TIMP-1 mRNA levels were increased by IL-1β in normoxic, hypoxic, and reoxygenated cultures, while those of TIMP-2 were not significantly altered in normoxia and hypoxia and were IL-1β–inhibited during oxidative stress.

We previously observed that TGFβ1 expression by chondrocytes was enhanced by IL-1β (34), and that blocking NO production led to further stimulation by the cytokine (Martin G, et al: unpublished observations). This indicates that NO exerts an inhibitory effect on TGFβ1 transcription. Here, we confirmed the IL-1β up-regulation of TGFβ1 expression in normoxia, but we found an inhibition by the cytokine in conditions of low oxygen tension and during oxidative stress. This finding is probably attributable to the higher level of NO produced by IL-1β action in these conditions, leading to an overall negative effect of the cytokine. The TGFβ2 gene promoter does not contain the same regulatory elements as TGFβ1 and, therefore, is not regulated in the same way by IL-1β (52). TGFβ2 protein was reduced by IL-1β only in normoxia, whereas no effect was observed in hypoxic and reoxygenated cultures.

TGFβRII plays a crucial role in the homeostasis of cartilage (53,54). Our present data clearly show that IL-1β down-regulates the expression of TGFβRII in both normoxia and hypoxia, suggesting that the cytokine could be responsible for the dramatic decrease in this receptor in OA cartilage (53). The enhanced expression of TGFβRI in hypoxia may potentially alter the ratio between the 2 receptors and also contribute to modulation of the TGFβ effect.

Finally, our findings indicate that oxygen tension can modulate the relative production of NO and PGE_2 induced by IL-1β in chondrocytes. NO production was significantly increased in hypoxic and reoxygenated cultures compared with normoxic controls, whereas the situation for PGE_2 synthesis was exactly the opposite. As an explanation for our results, it is likely that high levels of NO may exert an inhibitory effect on COX expression and activity, as previously reported (55,56). Our data are not in agreement with those of a recent study showing that porcine cartilage explants in hypoxia produced less NO under the effect of IL-1 than in normoxia (17). However, the culture system used by those authors had technical limitations and did not allow the experiments to be performed in conditions of continuous low oxygen tension, because culture media could not be replenished in hypoxic conditions.

In conclusion, culturing chondrocytes in a low-oxygen environment shows that the response of chondrocytes to IL-1, and probably to other cytokines and growth factors, is highly dependent on oxygen tension. Thus, some results previously obtained in normoxic cultures may be revisited to better understand the mechanisms of cartilage degradation in joint diseases. For example, the facts that NO is produced in greater amounts under the effect of IL-1 and that it also inhibits PGE_2 production support the view that NO may be the crucial IL-1–induced mediator in OA and RA (57). Studies are in progress to elucidate the mechanisms by which hypoxia and reoxygenation affect the response of chondrocytes to the cytokines and growth factors implicated in joint diseases.

REFERENCES

1. Shapiro IM, Mansfield KD, Evans SM, Lord EM, Koch CJ. Chondrocytes in the endochondral growth cartilage are not hypoxic. Am J Physiol 1997;272:C1134–43.
2. Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. Genes Dev 2001;15:2865–76.
3. Brighton CT, Heppenstall RB. Oxygen tension in zones of the epiphyseal plate, the metaphysis and diaphysis: an in vitro and in vivo study in rats and rabbits. J Bone Joint Surg Am 1971;53:719–28.
4. Silver IA. Measurement of pH and ionic composition of pericellular sites. Philos Trans R Soc Lond B Biol Sci 1975;271:261–72.
5. James MJ, Cleland LG, Rofe AM, Leslie AL. Intra-articular pressure and the relationship between synovial perfusion and metabolic demand. J Rheumatol 1990;17:521–7.
6. Mapp PI, Grootveld MC, Blake DR. Hypoxia, oxidative stress and rheumatoid arthritis. Br Med Bull 1995;51:419–36.
7. Ishikawa Y, Ito T. Kinetics of hemopoietic stem cells in a hypoxic culture. Eur J Haematol 1988;40:126–9.
8. Harrison JS, Rameshwar P, Chang V, Bandari P. Oxygen saturation in the bone marrow of healthy volunteers [letter]. Blood 2002;99:394.
9. Lewis JS, Lee JA, Underwood JC, Harris AL, Lewis CE. Macrophage responses to hypoxia: relevance to disease mechanisms. J Leukocyte Biol 1999;66:889–900.
10. Shapiro IM, Golub EE, Chance B, Piddington C, Oshima O, Tuncay OC, et al. Linkage between energy status of perivascular cells and mineralization of the chick growth cartilage. Dev Biol 1988;129:372–9.
11. Rajpurohit R, Koch CJ, Tao Z, Teixeira CM, Shapiro IM. Adaptation of chondrocytes to low oxygen tension: relationship between hypoxia and cellular metabolism. J Cell Physiol 1996;168:424–32.
12. Benya PD, Padilla SR, Nimni ME. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II: verification by cyanogen bromide peptide analysis. Biochemistry 1977;16:865–72.
13. Ysart GE, Mason RM. Responses of articular cartilage explant cultures to different oxygen tensions. Biochim Biophys Acta 1994;1221:15–20.
14. Grimshaw MJ, Mason RM. Bovine articular chondrocyte function in vitro depends upon oxygen tension. Osteoarthritis Cartilage 2000;8:386–92.
15. Grimshaw MJ, Mason RM. Modulation of bovine articular chondrocyte gene expression in vitro by oxygen tension. Osteoarthritis Cartilage 2001;9:357–64.
16. Murphy CL, Sambanis A. Effect of oxygen tension on chondrocyte extracellular matrix accumulation. Connect Tissue Res 2001;42:87–96.
17. Cernanec J, Guilak F, Weinberg JB, Pisetsky DS, Fermor B. Influence of hypoxia and reoxygenation on cytokine-induced production of proinflammatory mediators in articular cartilage. Arthritis Rheum 2002;46:968–75.
18. Pujol JP, Loyau G. Interleukin-1 and osteoarthritis. Life Sci 1987;41:1187–98.
19. Pelletier JP, Martel-Pelletier J. Evidence for the involvement of interleukin 1 in human osteoarthritic cartilage degradation: protective effect of NSAID. J Rheumatol Suppl 1989;18:19–27.
20. Maier R, Bilbe G, Rediske J, Lotz M. Inducible nitric oxide synthase from human articular chondrocytes: cDNA cloning and analysis of mRNA expression. Biochim Biophys Acta 1994;1208:145–50.
21. Palmer RM, Hickery MS, Charles IG, Moncada S, Bayliss MT. Induction of nitric oxide synthase in human chondrocytes. Biochem Biophys Res Commun 1993;193:398–405.
22. Stadler J, Stefanovic-Racic M, Billiar TR, Curran RD, McIntyre LA, Georgescu HI, et al. Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. J Immunol 1991;147:3915–20.
23. Knott I, Dieu M, Burton M, Houbion A, Remacle J, Raes M. Induction of cyclooxygenase by interleukin-1: comparative study between synovial cells and chondrocytes. J Rheumatol 1994;21:462–6.
24. Attur MG, Patel IR, Patel RN, Abramson SB, Amin AR. Autocrine production of IL-1β by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8. Proc Assoc Am Physicians 1998;110:65–72.
25. Lukiw WJ, Bazan NG. Strong nuclear factor-κB-DNA binding parallels cyclooxygenase-2 gene transcription in aging and in sporadic Alzheimer's disease superior temporal lobe neocortex. J Neurosci Res 1998;53:583–92.
26. Xie Q, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.
27. Eberhardt WC, Pliiss C, Hummel R, Pfeilschifter J. Molecular mechanisms of inducible nitric oxide synthase gene expression by interleukin-1β and cAMP in rat mesangial cells. J Immunol 1998;160:4961–9.
28. Mendes AF, Caramona MM, Carvalho AP, Lopes MC. Role of mitogen-activated protein kinases and tyrosine kinases on interleukin-1-induced NF-κB activation and iNOS expression in bovine articular chondrocytes. Nitric Oxide 2002;6:35–44.
29. Conca W, Kaplan PB, Krane SM. Increases in levels of procollagenase messenger RNA in cultured fibroblasts induced by human recombinant interleukin 1β or serum follow c-jun expression and are dependent on new protein synthesis. J Clin Invest 1989;83:1753–8.
30. Auble DT, Brinckerhoff CE. The AP-1 sequence is necessary but not sufficient for phorbol ester induction of collagenase in fibroblasts. Biochemistry 1991;30:4629–35.
31. Conca W, Auron PE, Aoun-Wathne M, Bennett N, Seckinger P, Welgus HG, et al. An interleukin 1β point mutant demonstrates that jun/fos expression is not sufficient for fibroblast metalloproteinase expression. J Biol Chem 1991;266:16265–8.
32. Rédini F, Mauviel A, Pronost S, Loyau G, Pujol JP. Transforming growth factor β exerts opposite effects from interleukin-1β on cultured rabbit articular chondrocytes through reduction of interleukin-1 receptor expression. Arthritis Rheum 1993;36:44–50.
33. Henrotin Y, Bruckner P, Pujol JP. The role of reactive oxygen

species in homeostasis and degradation of cartilage. Osteoarthritis Cartilage 2003;11:747–55.
34. Andriamanalijaona R, Felisaz N, Kim SJ, King-Jones K, Lehmann M, Pujol JP, et al. Mediation of interleukin-1β–induced transforming growth factor β1 expression by activator protein 4 transcription factor in primary cultures of bovine articular chondrocytes: possible cooperation with activator protein 1. Arthritis Rheum 2003; 48:1569–81.
35. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
36. Legendre F, Dudhia J, Pujol JP, Bogdanowicz P. JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of type II collagen, aggrecan core, and link protein transcription in articular chondrocytes: association with a down-regulation of SOX 9 expression. J Biol Chem 2003;278: 2903–12.
37. Bellido T, Borba VZ, Roberson P, Manolagas SC. Activation of the Janus kinase/STAT (signal transducers and activator of transcription) signal transduction pathway by interleukin-6-type cytokines promotes osteoblast differentiation. Endocrinology 1997; 138:3666–76.
38. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
39. Chadjichristos C, Ghayor C, Herrouin JF, Ala Kokko L, Suske G, Pujol JP, et al. Down-regulation of human type II collagen gene expression by transforming growth factor-β1 (TGF-β1) in articular chondrocytes involves SP1/SP3 ratio. J Biol Chem 2002;277: 43903–17.
40. Green L, Wagner D, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.
41. Serteyn D, Deby-Dupont G, Pincemail J, Mottart E, Philippart C, Lamy M. Equine postanaesthetic myositis: thromboxanes, prostacyclin and prostaglandin E2 production. Vet Res Commun 1988; 12:219–26.
42. Graven KK, McDonald RJ, Farber HW. Hypoxic regulation of endothelial glyceraldehyde-3-phosphate dehydrogenase. Am J Physiol 1998;274:C347–55.
43. Pfander D, Cramer T, Swoboda B, Johnson RS. HIF-1 α plays a critical role in growth-plate chondrocytes during low oxygen tension. Osteoarthritis Cartilage 2003;11 Suppl A:S5–S6.
44. Dudhia J, Wheeler-Jones C, Bayliss MT. Transient activation of p42/p44 MAPK by IL-1α is enhanced by a low oxygen tension in human articular cartilage. Proceedings of the 46th Annual Meeting, Orthopaedic Research Society; 2000 Mar 12-15; Orlando, FL.
45. Goldring MB, Tan L, Choy BK, Osaki M. Multiple interacting IL-1β-induced transcription factors converge to suppress constitutive COL2A1 promoter activity [abstract]. Osteoarthritis Cartilage 2002;10 Suppl A:S12.
46. Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor κB (NF-κB) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. Matrix Biol 2002;21:251–62.
47. Buttice G, Quinones S, Kurkinen M. The AP-1 site is required for basal expression but is not necessary for TPA-response of the human stromelysin gene. Nucleic Acids Res 1991;19:3723–31.
48. Pendas AM, Balbin M, Llano E, Jimenez MG, Lopez-Otin C. Structural analysis and promoter characterization of the human collagenase-3 gene (MMP13). Genomics 1997;40:222–33.
49. Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor κB: differential regulation of collagenase 1 and collagenase 3. Arthritis Rheum 2000;43:801–11.
50. Buttner FH, Chubinskaya S, Margerie D, Huch K, Flechtenmacher J, Cole AA, et al. Expression of membrane type 1 matrix metalloproteinase in human articular cartilage. Arthritis Rheum 1997;40:704–9.
51. Imai K, Ohuchi E, Aoki T, Nomura H, Fujii Y, Sato H, et al. Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinases 2. Cancer Res 1996;56:2707–10.
52. Noma T, Glick AB, Geiser AG, O'Reilly MA, Miller J, Roberts AB, et al. Molecular cloning and structure of the human transforming growth factor-β 2 gene promoter. Growth Factors 1991; 4:247–55.
53. Boumediene K, Conrozier T, Mathieu P, Richard M, Marcelli C, Vignon E, et al. Decrease of cartilage transforming growth factor-β receptor II expression in the rabbit experimental osteoarthritis: potential role in cartilage breakdown. Osteoarthritis Cartilage 1998;6:146–9.
54. Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, et al. Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol 1997; 139:541–52.
55. Amin AR, Attur M, Patel RN, Thakker GD, Marshall PJ, Rediske J, et al. Superinduction of cyclooxygenase-2 activity in human osteoarthritis-affected cartilage: influence of nitric oxide. J Clin Invest 1997;99:1231–7.
56. Fermor B, Weinberg JB, Pisetsky DS, Misukonis MA, Fink C, Guilak F. Induction of cyclooxygenase-2 by mechanical stress through a nitric oxide-regulated pathway. Osteoarthritis Cartilage 2002;10:792–8.
57. Clancy RM, Amin AR, Abramson SB. The role of nitric oxide in inflammation and immunity. Arthritis Rheum 1998;41:1141–51.