Regulation of Type II Collagen Synthesis during Osteoarthritis by Prolyl-4-Hydroxylases

Possible Influence of Low Oxygen Levels

Claudia Grimmer,* Nadine Balbus,* Ute Lang,* Thomas Aigner,[†] Thorsten Cramer,[‡] Lutz Müller,[§] Bernd Swoboda,* and David Pfander*

From the Division of Orthopedic Rheumatology,* Department of Orthopedic Surgery, University of Erlangen-Nuremberg, Erlangen; the Department of Pathology,[†] University of Leipzig, Leipzig; the Department of Hepatology and Gastroenterology,[‡] Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Berlin; and the Department of Orthopedic Surgery,[§] University of Erlangen-Nuremberg, Erlangen, Germany

Osteoarthritic (OA) chondrocytes are metabolically active, displaying increased synthesis of type II collagen. Here, we show by immunohistochemistry and polymerase chain reaction that in comparison with healthy cartilage, OA articular chondrocytes exhibit increased *in vivo* synthesis of collagen prolyl-4-hydroxylase type II, a pivotal enzyme in collagen triple helix formation. Exposure of primary human articular chondrocytes to 1% oxygen enhanced accumulation of native type II collagen and stabilized hypoxia-inducible factor-1 α (HIF-1 α). This effect was abolished by addition of the HIF-1 inhibitor 2-methoxyestradiol. Real-time polymerase chain reaction analyses of mRNAs from these cultures revealed increased transcript levels of both α -subunits of prolyl-4-hydroxylase (*P4HA1*, ~2-fold; *P4HA2*, ~2.3-fold) and of classical HIF-1 target genes (glucosetransporter-1, ~2.1-fold; phosphoglyceratekinase-1, ~2.2-fold). Treatment of hypoxic chondrocytes with 2-methoxyestradiol reduced transcriptional activity of HIF-1 and synthesis of α (II), and to a lesser extent α (I), subunits of collagen prolyl-4-hydroxylases. mRNA levels of type II collagen (*Col2A1*) and the β -subunit (*P4HB*) of prolyl-4-hydroxylase, however, displayed only modest changes at 1% oxygen. From these results and our *in vivo* data, we inferred that besides increased *Col2A1* mRNA expression by OA chondrocytes, accelerated posttranslational modification processes might contribute to the increased synthesis and accumulation of type II collagen during OA and experimen-

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Articular hyaline cartilage is the most important component in synovial joints for frictionless joint motion. During movement, the articular cartilage is compressed by load and is then able to recover from this deformity. These unique biomechanical properties depend on the specific zonal architecture of articular cartilage as well as the extracellular matrix (ECM), which consists of two phases: solid and fluid phases. The main ECM components are proteoglycans, glycoproteins, and collagens, mainly type II collagen.^{1–3} Type II collagen is synthesized as a procollagen molecule with noncollagenous amino and carboxy extension peptides, by articular chondrocytes which represent the only living elements within hyaline cartilage.⁴ Large type II collagen fibrils form a network with embedded proteoglycans, glycoproteins, water, and soluble ions. Since this collagen network is established in adults, the half-life of collagens is estimated to be over decades.

Proper function of a load-bearing joint depends on the structural integrity of this highly specialized cartilage tissue and its ability to absorb and respond to mechanical stress. During the course of osteoarthritis, increased degradation processes of the ECM by mechanical factors as well as activity of metalloproteinases and aggrecanases have been observed.^{5,6} Ultimately, destruction of large parts of the collagen network and ion-binding capacity via loss of negatively charged proteoglycans lead to a significantly decreased water-binding capability of the ECM. These biochemical changes are responsible for the devel-

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Address reprint requests to David Pfander, M.D., Division of Orthopedic Rheumatology, Department of Orthopedic Surgery im Waldkrankenhaus St. Marien, University of Erlangen-Nuremberg, Rathsbergerstrasse 57, D-91054 Erlangen, Germany. E-mail: dpfander@t-online.de.

opment of the biophysical hallmarks of OA cartilage: loss of shock-absorbing properties and soft structure.

However, besides the liberation of matrix destructive enzymes, osteoarthritic chondrocytes are metabolically active and increase gene expression of several matrix components, eg, collagens, glycoproteins, and small proteoglycans.⁷⁻⁹ This activity of OA chondrocytes is generally appreciated as an attempt to restore the ECM. In different studies with biochemical and molecular biology approaches, type II collagen synthesis was found to be increased between four- and sevenfold during OA.^{7,10-12} In addition, it was repeatedly shown in experimental OA and in affected human cartilage that the onset of type II collagen synthesis in OA is mainly localized in the deep zone.¹¹⁻¹³

In vitro, collagen II synthesis of bovine articular and murine epiphyseal chondrocytes is stimulated by hypoxia and indirectly controlled by the transcription factor hypoxia-inducible factor-1 α (HIF-1 α).¹⁴⁻¹⁶ Consistent with these data, we have further shown that accumulation of HIF-1 α by inactivation of its negative regulator, the von-Hippel-Lindau protein, led to increased extracellular matrix accumulation.¹⁷ The transcription factor HIF-1 α is the key molecule in adapting cells and tissues to hypoxic conditions responsible for the rapid synthesis of a set of proteins that enable cells to survive extremely hypoxic or anoxic environments. HIF-1 belongs to the family of basic helix-loop-helix-containing Per-Arnt-Sim motif transcription factors. HIF-1 is a heterodimeric protein consisting of HIF-1 β and HIF-1 α subunits.¹⁸ The active subunit α of HIF-1 confers the oxygen responsiveness and is hydroxylated by oxygen-sensitive prolylhydroxylases (PHD1 to PHD3) under ambient conditions, a process that initiates HIF-1 α degradation through the proteasome after targeting by the von-Hippel-Lindau protein and coupling to ubiquitin.¹⁹ It has been clearly established that HIF-1 is of fundamental importance in tumorigenesis, inflammation, ischemic processes, and growth-plate development.²⁰⁻²² Recently, evidence showed that 2-methoxyestradiol (2ME2), an endogenous metabolite of estrogen, is an inhibitor of HIF-1 α responsible for its potent antiangiogenic and antitumor effects *in vivo*.²³⁻²⁶

HIF-1 has been further described to be expressed by articular chondrocytes *in vivo*.^{27,28} This finding is of particular interest because it has been shown that the partial pressure of oxygen in synovial fluid is very low and is further decreased under pathological conditions such as OA.²⁹⁻³¹ In addition, Silver and colleagues have determined that oxygen levels vary from 7 to 10% in the superficial zone to values as low as 1% in the deep zone in healthy cartilage.³² In addition, we and other groups have demonstrated an increased number of chondrocytes staining for the transcription factor HIF-1 α and its target genes in OA cartilage samples.³³⁻³⁵ Despite a progressive loss of cartilage substance in OA leading to a decreased diffusion-distance of oxygen, an additional depletion of oxygen in osteoarthritic cartilage is reasonable to assume.

Two recent publications demonstrated in fibroblasts, vascular smooth muscle cells, and murine hepatoma cells that lowered oxygen levels increase the synthesis of

a group of procollagen hydroxylases that are necessary for the posttranslational hydroxylation of amino acid residues at the endoplasmatic reticulum.^{36,37} The most important members of these enzymes are collagen prolyl-4-hydroxylases (C-P4Hs), which catalyze the formation of 4-hydroxyproline, an essential step for collagen triple-helix formation. In humans, three isoforms of this $\alpha_2\beta_2$ -tetrameric enzyme exist, namely prolyl-4-hydroxylase I ($\alpha(I)_2\beta_2$), II ($\alpha(II)_2\beta_2$), and III ($\alpha(III)_2\beta_2$), which consist of different catalytic α -subunits and identical β -subunits identified as protein disulfide-isomerase.³⁸⁻⁴¹ Clear experimental evidence has been provided that prolyl-4-hydroxylase II is the main isoform expressed in epiphyseal chondrocytes.^{42,43} Thus, a contribution of collagen prolyl-4-hydroxylases and eventually their regulation through oxygen levels to the zone-specific synthesis pattern of type II collagen during OA can be hypothesized.

The aim of the present study was to characterize the expression pattern of prolyl-4-hydroxylases I and II in normal and OA human knee cartilage. In addition, we asked whether hypoxia via the transcription factor HIF-1 α directly regulates the expression of prolyl-4-hydroxylases and type II collagen *in vitro* compared with two classical HIF-1 target genes, glucose transporter-1 (*Glut-1*) and phosphoglycerate kinase-1 (*PGK-1*).

Materials and Methods

Patients and Samples for Immunohistochemistry

Cartilage/bone samples were obtained from patients who were hospitalized in the Division of Orthopedic Rheumatology, University Erlangen-Nuremberg, Germany. Clinical data included age, sex, patient history, physical examination, results of various blood tests (complete blood count, rheumatoid factors, erythrocyte sedimentation rate, serum electrophoresis, c-reactive protein, and other serum diagnostics), and x-rays of the respective knee joints. Data were carefully reviewed to exclude any secondary forms of OA and rheumatoid arthritis. Twenty-eight OA human knee cartilage samples were obtained from 25 donors during total knee replacement. Ten normal human cartilage samples were obtained from 10 knee joints of different donors during autopsies. The localization of the excised samples was carefully documented by video prints. The specimens were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), decalcified in diethylpyrocarbonate-treated 0.2 mol/L ethylenediamine tetraacetic acid, embedded in paraffin wax, and then cut into 6- μ m-thick sections perpendicular to the surface.

Histological-Histochemical Grading

Safranin-O-stained sections of normal and osteoarthritic cartilage specimens were graded according to Mankin et al⁴⁴ by two different observers. This histological-histochemical grading system ranging from 0 to 14 points analyzes structural abnormalities, cell population, safranin-O staining, and tidemark integrity in OA cartilage/

bone samples. Based on this grading system, samples were classified as normal (Mankin 0 to 1; $n = 10$), mild (Mankin 2 to 5; $n = 9$), moderate (Mankin 6 to 9; $n = 9$), and severe (Mankin >9 ; $n = 10$) osteoarthritic lesions as described in detail previously.^{45,46}

Immunohistochemistry

After deparaffinization, cartilage/bone slides were pretreated as follows. For immunostaining of $\alpha(I)$ -, $\alpha(II)$ -, and β -subunits of C-P4H, sections were pretreated with 10 mmol/L Tris-HCl (pH 10) at 95°C for 5 minutes and 1 mg/ml trypsin (Merck, Darmstadt, Germany) at 37°C for 15 minutes. For detection of type II collagen, sections were incubated with 2 mg/ml hyaluronidase (Merck) in phosphate-buffered saline, pH 5.5, for 15 minutes, followed by digestion with 1 mg/ml pronase for 30 minutes (Boehringer, Mannheim, Germany) in PBS (pH 7.5) as described previously.^{47,48} Nonspecific antibody binding was blocked with 5% bovine serum albumin in PBS, followed by washing in PBS. Then sections were incubated overnight at 4°C with polyclonal antibodies against $\alpha(I)$ - or $\alpha(II)$ -subunits (designed by J. Myllyharju, Oulu, Finland) in a dilution of 1:500 or 1:100, β -subunits (Acris Antibodies, Hiddenhausen, Germany) in a dilution of 1:40 or type-II collagen (MP Biomedicals Inc., Irvine, CA) in a dilution of 1:500 in 1% bovine serum albumin in PBS (pH 7.5). Control sections were incubated with an equal protein concentration of control IgGs or rabbit serum. After incubation with the first antibody, each step was followed by extensive washing in Tris-buffered saline (pH 7.5). Primary antibodies were then followed by incubation with biotinylated secondary antibodies diluted 1:500 for 1 hour at 37°C. A complex of streptavidin and biotin labeled with alkaline phosphatase was then added according to the protocol of the manufacturer (Dako, Hamburg, Germany). Antibody binding was visualized with Fast Red (Sigma, Munich, Germany). Finally, slides were counterstained with hematoxylin. For optimal staining results, several pretreatments including hyaluronidase, pronase, and trypsin were tested. The used pretreatment (Tris-HCl/trypsin) resulted in optimal staining results and was used for all slides. To determine the percentage of immunopositive chondrocytes, 100 cells were counted in each of two different areas throughout the entire depth of the respective 6- μ m-thick cartilage section under a high-power field. The average of the two counts was used for statistical analysis. Statistical analysis was performed on all data points with regard to immunopositive cells in normal articular cartilage by an unpaired Student's *t*-test. Data are given as mean $\pm$ SD. *P* values <0.05 were considered significant.⁴⁵

Double-Labeling Experiments for HIF-1 α and P4HA

For double immunodetection, 5 normal and 10 OA cartilage/bone slides were deparaffinized and pretreated as described above (Tris-HCl/trypsin). After nonspecific blocking, slides were incubated with a mixture of poly-

clonal anti- $\alpha(II)$ antibodies (designed by J. Myllyharju) diluted 1:50 and a monoclonal mouse-anti-HIF-1 α antibody diluted 1:200 (Novus Biologicals, Littleton, CO) overnight at 4°C. Specific binding were visualized by subsequently adding fluorescein isothiocyanate-labeled donkey anti-rabbit 1:200 and Cy3-labeled goat anti-mouse antibodies 1:200 (Dianova GmbH, Hamburg, Germany) for 3 hours at room temperature. Slides were covered with a 4'-6-diamidino-2-phenylindole-containing mounting medium (Vector Inc., Peterborough, UK).

RNA Isolation from Articular Cartilage

For polymerase chain reaction (PCR), total RNA was isolated from 10 osteoarthritic (grade II to III according to Outerbridge⁴⁹) and 9 normal cartilage samples as described in detail previously.¹⁰ Cartilage samples were obtained during knee replacement procedures or autopsies. For real-time PCR, total RNA yields were calculated by measurement of the extinction at 260 nm (UltrospecIII; Pharmacia LKB, Cambridge, UK). The quality of RNA isolation was controlled by using the Bioanalyzer RNA 6000 Pico LabChip (Agilent, Waldbronn, Germany).

Chondrocyte Cultures

Chondrocytes were cultured as described previously.⁹ In brief, human articular chondrocytes were isolated from the remaining articular cartilage of one knee joint during total knee replacements with two digestion steps. Severity of OA cartilage damage ranged from grade II to III according to the Outerbridge classification system.⁴⁹ First, articular cartilage was digested in Hanks' balanced salt solution containing 2 mg/ml pronase (30 minutes at 37°C) followed by 140 U/ml collagenase in Hanks' balanced salt solution (6 hours at 37°C). Chondrocytes were quantified using a hemocytometer. Chondrocytes (1.2 million) were plated per well in 6-well culture dishes in Dulbecco's modified Eagle's medium containing 10% bovine fetal serum, 2 mmol/L glutamine, and 50 U/ml streptomycin/penicillin. After 4 days, confluent chondrocytes were exposed 24 hours to hypoxia (1% oxygen) versus normoxia (20% oxygen), balanced with N₂ in a three-gas incubator in humidified atmosphere (Binder GmbH, Tuttlingen, Germany). To inhibit HIF-1 α activity under hypoxic conditions, 2ME2 was used in three concentrations of 10, 35, and 50 μ mol (Sigma).^{23,24} CoCl₂ in a final concentration of 50 μ mol was used to mimic a hypoxic environment. Then, whole-cell lysates and nuclear and cytoplasmic extracts were prepared. In addition, total RNA was harvested with the Nucleo-Spin-RNA-II kit (Clontech Laboratories, Palo Alto, CA) as recommended by the manufacturer.⁹ The quality of RNA isolation was controlled as described above. For type II collagen-protein measurements, chondrocytes were exposed to 1 versus 20% oxygen for 4 days.

Western Blotting

For Western blot analysis, nuclear and cytoplasmic extracts were prepared as described by Dignam et al.⁵⁰

Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). After boiling, 40 μ g protein of cytoplasmic fractions and nuclear extracts were separated on 10% sodium dodecyl sulfate polyacrylamide gels under reducing conditions and then transferred on nitrocellulose transfer membranes. To control equal loading, nitrocellulose membranes were stained with Ponceau-red. After blocking nonspecific binding sites with 5% low-fat milk powder in PBS, nitrocellulose membranes were incubated with a polyclonal anti-HIF-1 α antibody (R&D Systems, Wiesbaden, Germany) in a dilution of 1:1000 in 2.5% low-fat milk powder overnight at 4°C. Horseradish peroxidase-conjugated goat anti-rabbit antibodies (Dianova GmbH) diluted 1:10,000 were used as secondary antibodies. Then immunoreactive proteins were visualized using a chemiluminescence kit (Roche Diagnostics GmbH, Mannheim, Germany) followed by exposure to a chemiluminescent detection film (Roche Diagnostics GmbH). Prolyl-4-hydroxylase subunits 1 and 2 were detected in whole-cell lysates (50 μ g) using polyclonal antibodies at a dilution of 1:2000 (α (I)) or 1:500 (α (II)) (designed by Dr. J. Mlylyharju).

Immunofluorescence Staining

Treated chondrocytes were fixed with 4% paraformaldehyde. Cell layer was pretreated with 0.1% Triton-X in PBS for 15 minutes at room temperature. Nonspecific antibody binding was blocked with 5% bovine serum albumin in PBS, followed by washing in PBS. For immunodetection, a monoclonal mouse-anti-HIF-1 α antibody diluted 1:200 (Novus Biologicals) was used. Specific binding was visualized by adding Cy3-labeled goat anti-mouse antibodies 1:200 (Dianova GmbH) for 2 hours at room temperature. Stained chondrocytes were covered with a 4'-6-Diamidino-2-phenylindole-containing mounting medium (Vector Inc.).

Type II Collagen Enzyme-Linked Immunosorbent Assay (ELISA)

For collagen quantification, the native type II collagen detection kit was used (Chondrex Inc., Redmond, WA). Confluent primary chondrocytes were exposed to 4 days of hypoxia, normoxia, or 1% oxygen combined with 10 μ mol of 2ME2. The cell layer was washed twice in PBS. Then 0.5 ml of 0.05 mol/L acetic acid was added, and the cell layers were harvested by scraping. Cell suspension was transferred to a microcentrifuge tube, and 50 μ l of 1% pepsin solution in 0.05 mol/L acetic acid was added. Cell suspension was digested on a rotator overnight at 4°C. Then 50 μ l of Tris-saline buffer (1 mol/L Tris, 2 mol/L NaCl, and 50 mmol/L CaCl₂) was added to the suspension, and the pH was adjusted to 8.0. The 50- μ l 0.1% pancreatic elastase in TSB was added and digested for 30 minutes at 37°C. The digested suspensions were centrifuged at 10,000 $\times g$ for 5 minutes. The further type II collagen ELISA was conducted following the manufacturer's instructions. Type II collagen concentrations were

normalized to the total protein content, using the BCA protein assay (Pierce). For statistical analyses, the unpaired Student's *t*-test was calculated. Data are given as mean $\pm$ SD. *P* values <0.05 were considered significant.

Conventional Reverse Transcriptase-PCR (RT-PCR)

The RT reaction was performed using the first-strand cDNA synthesis kit (Roche Diagnostics GmbH). For conventional PCR, cDNAs from three normal and three OA cartilage samples were amplified through 28, 32, or 33 cycles, depending on the primer set used. The following primer sequences were used: β 2-microglobulin, forward 5'-GCTATCCAGCGTACTCCAAAGATT-3' and reverse 5'-CCACTTTTCAATTCTCTCTC-CATTC-3' (product 151 bp); *P4HA1*, forward 5'-GTGGATTACCTGCCAGAGAGACA-3' and reverse 5'-CTCGGCTCAGCCTTGTTT-3' (product 250 bp); *P4HA2*, forward 5'-CCTTGACCC-AAGCCACGA-3' and reverse 5'-CCTGTTGCCATGGTG-GTACC-3' (product 250 bp); *P4HB*, forward 5'-GGA-CGTGGAGTCGGACTCTG-3' and reverse 5'-GGCTGT-CTGCTCGGTGAACT-3' (product 250 bp); and *Col2A1* forward 5'-GGCAATAGCAGGTTACGTACA-3' and reverse 5'-CGATAACAGTCTTGCCCCACTT-3' (product 79 bp). Each PCR cycle involved denaturation at 94°C for 30 seconds, annealing, and primer extension at 60°C for 1 minute. PCR products of three healthy and three OA cartilage samples were analyzed on 2.5% agarose gels containing ethidium bromide as described previously.³⁴ DNA fragments of PCR products were sequenced. Homologies of sequenced nucleotides were analyzed using BLAST 2.2.1. All PCR products (β 2-microglobulin, *P4HA1*, *P4HA2*, *P4HB*, and *Col2A1*) revealed 97 to 100% homology to published nucleotide sequences of respective cDNAs.

Real-Time PCR

For real-time PCR analysis, RNAs from 9 healthy and 10 OA cartilage samples were compared. For measuring the effect of low oxygen tensions, RNAs from three or four independent cell-culture experiments were used. Quantitative real-time PCR was performed with an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) and QuantiTect Probe RT-PCR kit (Qiagen) for one-step RT-PCR. The results were captured and evaluated by use of SDS software (Applied Biosystems) and Microsoft Excel (Microsoft, Redmond, Washington). Relative quantification of gene expression with data from the detection system was performed using the standard curve method. For each sample, the relative amount of the target mRNA was determined and normalized to β 2-microglobulin. The primer/probe sets for collagen prolyl-4-hydroxylase subunits (*P4HA1*, *P4HA2*, and *P4HB*) and type II collagen (*Col2A1*) were purchased from Applied Biosystems/Celera Genomics (Table 1). The primer and probe sets for β 2-microglobulin,⁵¹ phosphoglyceratekinase-1, and glucosetransporter-1 were designed using the primer express software from Perkin Elmer, Wellesley, MA (Table 2). For statistical analyses, the unpaired Stu-

Table 1. Primer/Probe Sets of Target Genes Used for Real-Time PCR (TaqMan Assays-on-Demand Gene Expression Product, Applied Biosystems)

Symbol	Gene name	Gene expression assays	RefSeq
<i>P4HA1</i>	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), α -polypeptide I	Hs00168575_m1	NM_000917
<i>P4HA2</i>	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), α -polypeptide II	Hs00188349_m1	NM_004199
<i>P4HB</i>	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), β -polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	Hs00168586_m1	NM_000918
<i>COL2A1</i>	Collagen, type II, α 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	Hs00264051_m1	NM_033150 NM_001844

dent's *t*-test was calculated. Data are given as mean $\pm$ SD. *P* values <0.05 were considered significant.

Results

Prolyl-4-Hydroxylase Types I and II Are Detectable in Human Normal and OA Cartilage

Using immunohistochemistry and conventional PCR, we detected all components of prolyl-4-hydroxylase types I and II at the transcript and protein level. By testing different pretreatments with serial sections of normal and OA cartilage samples, we optimized the immunohistochemical staining procedure by using Tris-HCl at 95°C and trypsin digestion. We detected α (I)-, α (II)-, and β -subunits in all cartilage samples in a cellular and sometimes weak pericellular staining pattern (Figure 1). Furthermore, conventional PCR analyses confirmed mRNA expression of both α -subunits (*P4HA1* and *P4HA2*) and the single β -subunit (*P4HB*) of C-P4H (Figure 2). In human normal cartilage, chondrocytes positively stained for the α (II)-subunit were detectable mainly in the deep zone, where the lowest oxygen levels have been proposed (Figure 1C). In a previous study, we immunodetected HIF-1 α -positive chondrocytes within deep and middle zones of normal articular cartilage.³³ To test whether distribution of α (II)-subunits correlates to the staining of the transcription factor HIF-1 α , double-immunofluorescence staining was conducted. These experiments showed α (II) staining mainly in those articular chondrocytes displaying a nuclear, mixed nuclear/perinuclear, or perinuclear HIF-1 α accumulation (Figure 3B). In cartilage samples with mild, moderate, and severe OA changes, significantly increased numbers (*P* < 0.01) of α (II)-subunit-positive chondrocytes were determined (Figures 1, H and M, and 3A). In OA cartilage specimens with severe OA changes, the percentage of α (II)-positive chondrocytes was significantly increased to 39 compared with 9% stainable cells in normal articular cartilage

(Figure 3A). Furthermore, double labeling revealed that α (II) expression was colocalized to the transcription factor HIF-1 α in chondrocyte clusters from severely affected cartilage (Figure 3B). Chondrocytes stainable for the α (I)-subunit were distributed in the upper zone and throughout the entire depth of healthy articular cartilage (Figure 1B). In cartilage samples with severe OA changes, percentages of α (I)-positive chondrocytes increased significantly (27% in severe versus 10.5% in normal cartilage, *P* < 0.01) compared with percentages of positive cells in normal samples (Figures 1, G and L, and 3A). In contrast, no significant differences in the presence and distribution of β -subunits of C-P4H were detectable in healthy and OA joints by means of immunohistochemistry (Figures 1, D, I, and N, and 3A). High numbers of articular chondrocytes distributed throughout all cartilage zones showed positive immunostaining for β -subunits (protein disulfide-isomerase) of C-P4H. However, a modest increase in the number of β -subunit-positive chondrocytes was determined (73.6% in severe OA versus 55.2% in normal cartilage, *P* > 0.05). This observation is well in line with the idea that protein disulfide-isomerase is present in excess compared with corresponding α -subunits in many tissues. In late-stage cartilage disease, all subunits of prolyl-4-hydroxylase types I and II were detectable in the majority of OA chondrocytes of remaining middle and deep zones (Figure 1, L–O). In these late OA stages, collagen synthesis has been shown to be strongly activated, as indicated by an increased territorial type II collagen deposition (Figure 1O).^{11,52} Control slides incubated with equivalent concentrations of mouse IgGs or rabbit serum for control purposes revealed no staining (not shown).

Prolyl-4-Hydroxylase Type II and Type II Collagen Gene Expressions Are Up-Regulated during Osteoarthritis

In the next set of our experiments, we quantified gene expression levels of the three prolyl-4-hydroxylase sub-

Table 2. Sequences of Designed Primers and Probes

Genes	Forward	Reverse	Probe
<i>Glut-1</i>	GCCTGGATCTCCCCACTCTAG	CAGGTAAAGAAAGATTAATTTGAGTGGTT	ATTAGGATTTGCCCTTCCCAT
<i>B2M</i>	TGACTTTGTACAGCCCAAGATA	AATCCAAATGCGGCATCTTC	TGATGCTGCTTACATGTCTCGATCCCA
<i>PGK-1</i>	AAGAAGGGAAGGAAAAGATGCT	ACATCCCTTAGCTTGAAAGTG	TGGGAACAAGGTTAAAGCCGAGCCA

Figure 1. Cartilage/bone sections of normal (A–E) and OA (F–O) joints are demonstrated. **A:** Healthy articular cartilage (safranin-o staining). **B:** Showing chondrocytes stained for $\alpha(I)$ -subunits of C-P4H (I) at the surface and throughout all cartilage layers. **C:** Positive chondrocytes immunostained for $\alpha(II)$ -subunits are located mainly in the deep zone (arrowheads). **D:** Immunostaining for β -subunits of C-P4H reveals positive chondrocytes distributed throughout all cartilage layers. **E:** Type II collagen deposition is seen in all cartilage layers. **F:** Mild OA lesion with a decreased proteoglycan staining and fissuring of the surface of the upper cartilage (safranin-o staining). **G:** $\alpha(I)$ -immunostaining of a mild OA sample (indicated by arrowheads). **H:** Immunostaining displays $\alpha(II)$ -positive chondrocytes in all cartilage layers (indicated by arrowheads). **I:** Immunodetection of β -subunits in mild OA cartilage. **J:** Immunohistochemical analysis of type II collagen shows the appearance of a territorial “halo” around the chondrocytes of the middle and deep zone, suggesting degradation of type II collagen in the territorial matrix. **K:** Proteoglycan staining of severely affected cartilage. **L–N:** Immunostaining for all components of C-P4H types I and II showing high numbers of positive chondrocytes, specifically in chondrocyte clusters ($\alpha(I)$ -, $\alpha(II)$ -positive areas are indicated by arrowheads). **O:** Immunostaining for type II collagen shown in the same section presented in **K–O** displaying a strong territorial deposition of collagen II, indicating an increased synthesis (indicated by arrowheads). Scale bar: 200 μ m (A–E); 300 μ m (F–O).

units as well as type II collagen in normal and OA cartilage samples by real-time PCR. As demonstrated in Figure 2, we could detect highly significant increased gene expression levels of $\alpha(II)$ -subunits (*P4HA2*) and β -subunits (*P4HB*) (~ 2.2 -fold, $P < 0.01$) in OA compared with healthy joints (normalized to $\beta 2$ -microglobulin). However, *P4HA2* gene expression (> 8 -fold, $P < 0.01$) displayed a more substantial up-regulation when compared with *P4HB* mRNA expression (Figure 2B). The more than twofold increased transcript levels of *P4HB* in OA samples (versus normal samples) are confirming our immunohistochemically data, showing a slightly increased number of β -subunit-positive chondrocytes from 55.2% to more than 70% in cartilage specimens with mild (71.7%), moderate (71.5%), and severe (73.6%) OA changes. mRNA expression levels of the $\alpha(I)$ -subunit (*P4HA1*) showed a modest but not statistically significant increase (Figure

2A). Finally, type II collagen mRNA levels increased significantly (~ 4.7 -fold, $P < 0.05$) during the course of osteoarthritis (Figure 2D), a result well in line with earlier reports by several groups.¹⁰

Native Type II Collagen Content Is Increased under Hypoxia in the Presence of HIF-1 α

We and other groups have previously shown that type II collagen levels in murine chondrocyte cultures are increased in hypoxic environments, a phenomenon eventually mediated by the transcription factor hypoxia-inducible factor-1 α .^{14,15} Consistent with this result, we have further shown that accumulation of HIF-1 α by inactivation of its negative regulator von-Hippel-Lindau protein led to increased extracellular matrix accumulation.¹⁷ In this study, we cultured human primary articular chondrocytes

Figure 2. Results of conventional PCR (top panels) and real-time PCR analyses (bottom graphs) of mRNAs from healthy and OA cartilage samples are demonstrated. **A–D:** PCR products from three normal and three OA RNA extracts confirm gene expression of *P4HA1* (250 bp, 32 cycles), *P4HA2* (250 bp, 32 cycles), *P4HB* (250 bp, 28 cycles), and *Col2A1* (79 bp, 33 cycles) in normal and OA cartilage (compared with β 2-microglobulin, 151 bp, 33 cycles). Bars represent the severalfold changes of *P4HA1* (**A**), *P4HA2* (**B**), *P4HB* (**C**), and *Col2A1* (**D**) mRNA levels (normalized to β 2-microglobulin) in OA ($n = 10$) compared with healthy cartilage samples ($n = 9$). Bars represent means ($\pm$ SD). * $P < 0.05$, ** $P < 0.01$ OA versus healthy samples.

in a high-density monolayer culture system. As demonstrated in Figure 4A, more than threefold increased type II collagen contents were detectable in hypoxic chondrocyte cultures by ELISA ($P < 0.01$). Next, we questioned whether these increased collagen contents in hypoxic

chondrocytes cultures depend on the presence of the transcription factor HIF-1 α . Immunoblotting showed a stabilization and nuclear accumulation of HIF-1 α after exposition to 1% oxygen and treatment of CoCl₂, which mimics the effect of hypoxia by inhibition of HIF-1-de-

Figure 3. A: Percentages of chondrocytes in normal, mild, moderate, and severe OA cartilage specimens showing immunostaining for α (I)-subunits, α (II)-subunits, and β -subunits of collagen prolyl-4-hydroxylase types I and II. One hundred cells were counted in each of two different areas throughout the entire depth of the cartilage specimens under a high-power field. The average of the two counts was used for statistical analysis. Statistical analysis was performed on all data points with regard to immunopositive cells in normal articular cartilage by an unpaired Student's *t*-test. Data are given as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ (normal (norm.) versus mild OA, norm. versus moderate OA, norm. versus severe OA). **B:** Double-immunofluorescence staining for the α (II)-subunit and HIF-1 α in normal (top panels) and OA cartilage (bottom panels). Arrowheads are indicating nuclear staining of HIF-1 α . Scale bar = 30 μ m.

Figure 4. **A:** Quantification of type II collagen in primary human chondrocyte cultures. After confluency, chondrocytes were exposed to 21% oxygen, 1% oxygen, or 1% oxygen and treated with 10 μ mol of 2ME2 for 4 days. The collagen contents of normoxic chondrocyte cultures were set as 1. Data were obtained from three different experiments. Values are expressed as mean ($\pm$ SD). * P < 0.05, ** P < 0.01 norm. versus hypoxia (hyp.) or hyp. versus hyp. + 2ME2. **B:** Western blot analysis of HIF-1 α in primary human chondrocytes. Human chondrocytes were cultivated in normoxia (21%), hypoxia (1%), and normoxia with CoCl₂ (50 μ mol), which mimics the effect of hypoxia by inhibitions of prolylhydroxylases. To analyze the effect of HIF-1, inhibiting 2ME2 cytoplasmatic fractions and nuclear extracts of chondrocytes exposed to 1% O₂ and 1% O₂ treated with 35 μ mol of 2ME2 were harvested (bottom blot). Western blots were probed with a polyclonal-anti-HIF-1 α -antibody (R&D Systems) in a dilution of 1:1000. Equal loading and blotting were controlled by Ponceau-red staining. **C:** Immunofluorescence staining for HIF-1 α revealed a nuclear translocation of HIF-1 α and 1% oxygen. This hypoxic effect was abolished by an additional treatment 35 μ mol of 2ME2.

grading prolylhydroxylases (Figure 4B). In contrast, hypoxia-inducible stabilization and nuclear accumulation of the transcription factor was abolished by treatment with 2ME2, a recently described potent inhibitor of HIF-1 α activity (Figure 4, B and C).^{23,24} To avoid 2ME2 effects on chondrocyte death by complete inactivation of HIF-1 α during the 4-day exposition time to 1% oxygen, a lowered 10- μ mol 2ME2 concentration, which does not affect cell

density and vitality (D.P., C.G., N.B., B.S., unpublished observation), was used for collagen experiments. By adding 10 μ mol of 2ME2, the hypoxia-dependent increase of type II collagen contents was completely lost. These results strongly suggest that HIF-1 α , probably through its control over prolyl-4 hydroxylases, is involved in the hypoxic increase of type II collagen synthesis by articular chondrocytes.

Figure 5. A: Bars are representing the severalfold changes of *P4HA1*, *P4HA2*, *P4HB*, *Col2A1*, *PGK-1*, and *Glut-1* mRNA levels (normalized to β 2-microglobulin) compared to normoxia (onefold expression). Primary chondrocytes were cultured under hypoxic conditions and treated with 2ME2. Data were obtained from seven independent experiments. Bars represent means ($\pm$ SD). * P < 0.05, ** P < 0.01 normal versus hypoxia (hyp.) or hyp. versus hyp. + 2ME2. **B:** Western blot analysis for α (I)- and α (II)-subunits of C-P4H in primary human chondrocytes. Human chondrocytes were cultivated in normoxia (21%), hypoxia (1%), and hypoxia with 2ME2 (35 μ mol), which inhibits HIF-1 activity. Western blots were probed with polyclonal-anti- α (I)- or α (II)-antibodies (designed by Dr. J. Myllyharju, Oulu, Finland) in a dilution of 1:2000 (α (I)) or 1:500 (α (II)). Equal loading and blotting were controlled by Ponceau-red staining.

Gene Expression of α -Subunits Is Increased by Low Oxygen Tension

As shown in Figure 5, mRNA levels of both α -subunits of C-P4H were significantly induced in primary chondrocyte cultures exposed to low oxygen levels in a manner comparable with the well-established HIF-1 α target genes glucose transporter-1 and phosphoglycerate kinase-1. The comparable increase of *P4HA1* (~2-fold, P < 0.05), *P4HA2* (~2.3-fold, P < 0.01), *Glut-1* (~2.1-fold, P < 0.01), and *PGK-1* (~2.2-fold, P < 0.05) transcripts under 1% oxygen suggests that even *P4HA1* and *P4HA2* gene expression is controlled by the transcription factor HIF-1 (Figure 5A). Treatment of hypoxic chondrocytes with 2ME2 leads to a dose-dependent decrease of hypoxia-induced gene expression. A similar regulated synthesis of α (I)- and α (II)-subunits under hypoxia was detectable by immunoblotting (Figure 5B). However, hypoxic induction of α (II)-synthesis was more pronounced compared to synthesis of α (I)-subunits. Even chondrocytic *P4HB* and *Col2A1* mRNA levels were slightly increased after exposure to 1% oxygen (P < 0.05) and down-regulated by 2ME2 when normalized to β 2-microglobulin.

Discussion

This is the first study describing gene and protein expression of collagen prolyl-4-hydroxylases in human normal and OA cartilage by means of immunohistochemistry and conventional and real-time PCR. Increased collagen synthesis specifically in the deeper OA cartilage is a central hallmark of osteoarthritis.^{5,7,11,52} Collagen synthesis is a multiple step process consisting of collagen gene expression, translation, posttranslational modification, triple-helix formation, secretion, and cleavage of N- and C-propeptides in the extracellular space. Collagen prolyl-4-hydroxylase types I and II, which are highly expressed in chondrocytes, play a major role in the synthesis of type II collagen because of their ability to hydroxylate proline residues indispensable for collagen triple helix formation and efficient procollagen secretion. A recent study by Hofbauer et al³⁷ has shown that hypoxic conditions lead to increased expression of procollagen hydroxylases by activation of the transcription factor HIF-1 α . We have recently shown that HIF-1 α is essential for chondrocyte energy generation, thus increasing matrix formation and collagen synthesis *in vivo* and *in vitro*.^{13,33} In addition, we

have provided experimental evidence that HIF-1 α activity is increased in OA cartilage, probably resulting from the lowered oxygen levels in synovial fluids characterizing this disease.³³ Based on these observations, we questioned whether collagen prolyl-4-hydroxylase is synthesized in human normal cartilage and whether its expression pattern changes during progression of OA and under hypoxic conditions.

We detected chondrocytes staining for the α (II)-subunit of C-P4H predominantly in deep zones of healthy cartilage in a cellular and rarely pericellular staining pattern. Immunostaining for the α (II)-subunit colocalizes to HIF-1 α in articular cartilage, an observation that suggests a role of HIF-1 α in controlling *P4HA2* gene expression *in vivo*. HIF-1 α immunostaining was heterogeneously detectable in nuclear, mixed nuclear/perinuclear, or perinuclear staining patterns, as described in bovine chondrocytes or other malignant cell types.^{53,54} In contrast, a small number of α (I)-stained and a great number β -subunit-positive chondrocytes were distributed in all cartilage zones of normal and OA cartilage samples. Synthesis of α (I)-, α (II)-, and β -subunits of C-P4H was confirmed by conventional PCR and subsequent sequencing of the resulting PCR-products. In mRNAs from OA cartilage specimens, real-time PCR analyses revealed increased gene expression levels of type II collagen and to a lesser degree of *P4HB* compared with mRNA extracts from healthy articular cartilage. The most pronounced increase in mRNA expression during the course of OA was noted for *P4HA2*, a component of the heterotetrameric prolyl-4-hydroxylase type II, the main enzymatic isoform in chondrocytes.⁴³ Even significantly increased numbers of chondrocytes stainable for α (II)-subunits of C-P4H were present in samples from OA cartilage and distributed evenly in the remaining middle and deep layers of degenerated cartilage. The observed high percentages of chondrocytes immunostained for β -subunits and the real-time PCR results strongly suggest that β -subunits of C-P4H are synthesized in excess compared with corresponding α -subunits in healthy and diseased articular cartilage. From these results, we inferred that increased collagen prolyl-4-hydroxylase activity in OA cartilage depends on the increased synthesis of the catalytic α (II)-subunits, which then form the heterotetrameric prolyl-4-hydroxylase II. This assumption is underlined by previous studies, having shown that C-P4H type I is only a minor component in chondrocytes and cartilage tissues.^{42,43} Our investigations in human articular cartilage are further supported by a previous report having shown an enhanced enzyme activity of C-P4H in primary chondrocytes cultured from arthritic rat cartilage.⁵⁵

Increased type II collagen mRNA levels as an indicator of a general metabolic activation of OA chondrocytes have been repeatedly demonstrated by independent research groups and are widely assumed to be the major cause for activated collagen II synthesis during OA. Our experiments now show evidence for an enhanced synthesis of collagen prolyl-4-hydroxylases during the development of OA, suggesting posttranslational modification processes as an additional causal factor of increased type II collagen accumulation in degenerative cartilage diseases.

Given the heterogeneous distribution of oxygen in normal articular cartilage and the appearance of extremely hypoxic areas in deep zones of healthy and probably more diffuse in OA cartilage, we analyzed the influence of *in vitro* hypoxia for type II collagen levels and HIF-1 α accumulation in primary articular chondrocyte cultures. As shown in murine and bovine chondrocytes, exposure to low oxygen levels leads to a significantly increased type II collagen amounts in three independent cultures from different donors.^{14–16} Increased collagen accumulation induced by 1% oxygen was accompanied by stabilization, nuclear translocation, and increased activity of the transcription factor HIF-1 α . Inhibition of HIF-1 activity with 2-methoxyestradiol resulted in reduced nuclear HIF-1 α levels and strongly reduced type II collagen amounts, showing that HIF-1 activity is a necessary factor for collagen synthesis.

Finally, we investigated whether hypoxia influences mRNA levels of prolyl-4-hydroxylase subunits, *PGK-1*, *Glut-1*, and type II collagen. Transcript levels of *P4HA1* (~2-fold) and *P4HA2* (~2.3-fold) were significantly induced by exposure to 1% oxygen in a manner comparable with two well-known HIF-1 α target genes: glucose transporter-1 and phosphoglycerate-kinase-1 (*Glut-1*, ~2.1-fold; *PGK-1*, ~2.2-fold). To test whether hypoxic effects on gene expression are mediated by HIF-1 α , again 2-methoxyestradiol was used.^{23,24} Inhibition of HIF-1 accumulation led to a complete loss of hypoxia-induced gene expression of *Glut-1*, *PGK-1*, and *P4HAs*, suggesting that really HIF-1 α transactivates the respective genes in articular chondrocytes. The observed modest increase of type II collagen mRNA (1.5-fold) under hypoxia was well in line with our previous results in murine growth plates, displaying an increased matrix accumulation caused by an enhanced activity of the transcription factor HIF-1.¹⁷ However, despite the absence of a HIF-1 α -binding site within the collagen II gene, treatment with 2-methoxyestradiol leads to a significant reduction of *Col2A1* mRNA levels during low oxygen levels. As shown previously in epiphyseal chondrocytes, the effects of hypoxia and HIF-1 α on *Col2A1* expression might be more indirect via an increased and highly efficient ATP generation through the glycolytic pathway in the presence of glucose. Furthermore, the minor increase in *Col2A1* mRNA levels is not sufficient to explain the more than threefold increased type II collagen protein levels in hypoxic chondrocyte cultures. Rather, a HIF-1 α -dependent improvement in anaerobic energy generation and an increased synthesis of collagen prolyl-4 hydroxylase type II and to a lesser degree type I might be additional and reasonable candidates to accelerate type II collagen synthesis in osteoarthritis *in vivo* and in hypoxic environments *in vitro*.

mRNA expression levels of the β -subunit were slightly stimulated by hypoxia but repressed by additional treatment with 2ME2. The low induction of hypoxic *P4HB* mRNA expression stands in contrast to those described by Hofbauer et al³⁷ in mouse embryonic fibroblasts and murine hepatoma cells. However, the degree of hypoxic induction of *P4HB* described by Hofbauer et al³⁷ was strongly dependent on cell type and time point after stimulation with hypoxia. Therefore, our results support

the widely accepted hypothesis that even in cartilage tissues, the β -subunit of C-P4H is present in excess and its synthesis just slightly up-regulated during OA eventually resulting from the general metabolically activation of chondrocytes during OA.

Considering the fact that hypoxia, which might be more pronounced during the osteoarthritic process, leads to an increased synthesis of oxygen-consuming collagen prolyl-4-hydroxylase type II, we have to question whether this up-regulation is solely a compensatory mechanism. Alternatively, lowered oxygen levels lead to increased synthesis of native type II collagen, as our cell culture experiments suggested, probably in concert with the up-regulation of oxygen-binding proteins able to deliver oxygen to the collagen-synthesizing cells. Furthermore, the regulatory function of HIF-1 α in chondrocytes for anaerobic energy generation might be indirectly involved in controlling matrix synthesis, as demonstrated in murine growth-plate chondrocytes.¹³ However, our previous studies and the herewith presented data support the idea that even the increased synthesis of hypoxia-inducible collagen prolyl-4-hydroxylase type II might contribute to the increased type II collagen synthesis during OA.³³ In addition, our previous work and the observation that newly synthesized type II collagen is mainly found in the lowest deep zone of healthy cartilage and remaining middle and deep zones of OA cartilage strengthen the idea that the lowered oxygen levels in OA lead to an increased HIF-1-dependent transcription of *P4HA2*, which then forms the active heterotetrameric enzyme together with protein disulfide-isomerase.^{7,11,12,28,33} One might therefore hypothesize that these HIF-1 α -dependent mechanisms together with increased gene expression of type II collagen constitute important components in a combined effort to repair the ECM during OA. However, our data cannot exclude the possibility that the observed qualitative and quantitative changes in chondrocytic synthesis pattern during OA are influenced by other important mechanisms such as mechanical load, pro-inflammatory cytokines, growth factors, and differentiation processes. In this context, it is noteworthy that besides low oxygen levels, pro-inflammatory cytokines and mechanical overload, which are known to be important pathogenically factors in cartilage destruction, have been shown to increase HIF-1 α activity in bovine and human articular chondrocytes.^{56–58}

Nevertheless, in view of the decreased oxygen levels in OA synovial fluids and the herewith presented data, an important role of HIF-1 and prolyl-4-hydroxylase activity in cartilage biology and OA is likely to exist. Further studies are needed to outline the detailed molecular mechanisms underlying the role of HIF-1 α and C-P4H in controlling metabolic activity and matrix synthesis of articular chondrocytes under normal and pathological conditions such as osteoarthritis.

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