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Role of hypoxia-inducible factor-1 alpha in the regulation of plasminogen activator activity in rat knee joint chondrocytes

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Summary

Objective: To examine the effects of hypoxia-inducible factor-1α (HIF-1α) on the plasminogen activator's (PA) activity and on the expression of components of PA system in articular chondrocytes of rats.

Methods: Chondrocytes from rat knee joint cartilage were cultured under normoxic, hypoxic, $CoCl_2$ simulated hypoxic, and interleukin-1β (IL-1β)-stimulated conditions. siRNA targeting HIF-1α was transfected into cells cultured under hypoxic, simulated hypoxic, and IL-1β-stimulated conditions to silence HIF-1α. PA activity was determined by the hydrolysis of the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251). The mRNA levels were measured by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The intracellular/matrix-associate protein levels were detected by Western blot and the soluble protein levels were measured by enzyme linked immunosorbent assay (ELISA). Chromatin immunoprecipitation (CHIP) assay was performed to determine whether HIF-1α binds to the hypoxia response element (HRE) of target genes.

Results: The enhancement of HIF-1α by $CoCl_2$ resulted in a decrease of PA activity, and the silence of HIF-1α by siRNA led to an increase of PA activity. The PA inhibitor-1 (PAI-1) mRNA and protein were increased by hypoxia or simulated hypoxia, which was reversed by the siRNA2-mediated silencing of HIF-1α. CHIP assay further confirmed that the induction of PAI-1 involved the binding of HIF-1α to the PAI-1 promoter, while the enhancement or silencing of HIF-1α did not affect the expression of urokinase type PA (uPA), tissue type PA (tPA) or uPA receptor (uPAR). Additionally, IL-1β stimulated both HIF-1α and PAI-1 in articular chondrocytes, and the IL-1β-mediated induction of PAI-1 was inhibited partly by HIF-1α silencing.

Conclusion: HIF-1α may inhibit the PA activity through stimulating the expression of PAI-1 in normal articular chondrocytes. The inhibition of HIF-1α in the PA activity of articular chondrocytes probably plays an important role in the maintenance of articular cartilage matrix.

Key words: Hypoxia-inducible factor-1α, Plasminogen activator, Cartilage, Chondrocytes.

Introduction

The plasminogen activator (PA) system is mainly composed of urokinase type PA (uPA), tissue type PA (tPA), uPA receptor (uPAR), and PA inhibitor-1 (PAI-1). Both uPA and tPA are serine proteases that catalyze the conversion of the proenzyme plasminogen (PLG) to the broad-spectrum serine proteinase plasmin (PL)[1] that degrades the extracellular matrix (ECM) directly or indirectly through activation of secreted matrix metalloproteinases (MMPs)[2] PA is intensified and activated through binding to a specific cell-bound receptor uPAR, which is expressed on a variety of cell types, including chondrocytes[3] while PA is inactivated by its specific inhibitor PAI-1[4] There is a growing body of evidence to suggest that the PA system plays an important role in the degradation procedure of osteoarthritis (OA)[5–8]. Increased levels of PA system have been detected in OA cartilage lesions[5,6], and the PA dependent cartilage destruction has been demonstrated by several *in vitro* studies[7,8]. The recent appliance of gene-deficient mice showed that PLG deficiency leads to less severe arthritis than wild-type mice using collagen-induced arthritis model[9,10]. Besides, clinical improvement of patients with OA was observed after intra-articular injection of urinary trypsin inhibitor, which was shown to inhibit uPA[11]. All these observations support the idea that the PA system is mainly detrimental to cartilage integrity.

Within synovial joints, oxygen supply to articular chondrocytes is very limited and depends on the dynamic flow of synovial fluid during joints movement[12,13]. It has been demonstrated that an oxygen gradient exists in cartilage from around 6% at the joint surface to 1% in the deep layers[14]. The chondrocytes live in extreme micro-environmental conditions facing continuous low oxygen tensions. However, chondrocytes have been shown to be well adapted to these conditions[15,16]. Recently, the hypoxia-inducible

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Table I
siRNA targeting HIF-1α designed and chemically synthesized for transfection to chondrocytes

HIF-1α siRNA	siRNA ID	Targeted exon(s)	Sequence (5′–3′)	
			Sense	Antisense
siRNA1	52882	2, 3	GGAAACUUCUAGGUGCUGGtt	CCAGCACCUAGAAGUUUCCtt
siRNA2	52968	14	GGAUACAUAUCUAGUGAACtt	GUUCACUAGAUAUGUAUCCtt
siRNA3	199319	9	CCAGUUGAAUCUUCAGAUAtt	UAUCUGAAGAUUCAACUGGtt

factor-1α (HIF-1α) has been reported to play a major role in mediating the transcriptional response to hypoxia[17], leading to the survival of mammal cells under low oxygen tension. The relatively high constitutive expression of HIF-1α by chondrocytes may be an important adaptation to survival in the avascular-hypoxic environment of cartilage[18].

A previous study conducted on rat hepatocytes has shown that HIF-1α regulates the expression of PAI-1 through binding to the hypoxia response element (HRE) of PAI-1[19]. However, no study, to our knowledge, has investigated whether the PA activity is regulated by HIF-1α in articular chondrocytes.

Additionally, proinflammatory cytokines are believed to play a pivotal role in the initiation and development of OA process, among which IL-1β appears prominent. It has been reported that IL-1β may regulate the expression of PA system in several cell types[20,21]. Interestingly, IL-1β has also been suggested to lead to an up-regulation of HIF-1α in OA chondrocytes[22]. However, whether HIF-1α participates in the IL-1β-mediated regulation of PA system also remains unclear in articular chondrocytes.

Thus, the present study was carried out to elucidate the effects of HIF-1α on PA activity and the expression of PA system, as well as the role of HIF-1α in the IL-1β-mediated regulation of PA system.

Materials and methods

CHONDROCYTES ISOLATION AND CULTURE

Chondrocytes were isolated from articular cartilage of the knee joints of 5-week-old Sprague-Dawley (SD) rats as described previously[23]. In brief, articular cartilage layers of the knee joints were minced into small pieces, washed in phosphate buffered sodium (PBS), and subsequently digested in 0.25% trypsin containing ethylene diamine tetraacetic acid (EDTA) for 30 min and then in 0.2% collagenase II (Sigma, St. Louis, MO, USA) for 3 h at 37°C. Chondrocytes were washed in phosphate buffered sodium (PBS, centrifuged, and then seeded at a density of 3×10^4 cells/cm^2 in 25-cm^2 flasks. The cells were grown in monolayer cultures in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 25 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid (HEPES), and 100 units/ml penicillin and streptomycin in a humidified 5% CO_2 atmosphere. The cells were cultured under 2% (hypoxia)[24] or 20% (normoxia) oxygen, respectively, balanced with N_2 in a 3-Gas incubator (Binder, Tuttlington, Germany) in a humidified atmosphere. In a simulated hypoxic condition, $CoCl_2$ (150 μM; Sigma), a chemical inducer of HIF-1, was added to the cells incubated under normoxia. To evaluate the effects of IL-1β, cells were treated with 10 ng/ml of IL-1β (R&D Systems, Minneapolis, MN, USA)[22].

HIF-1α siRNA SYNTHESIS AND TRANSFECTION

Pre-designed siRNAs (cat#AM16708A) targeting rat HIF-1α were designed and chemically synthesized by Ambion (Austin, TX, USA), and three different siRNAs (Table I) from different regions of HIF-1α gene were selected. A non-targeting scramble siRNA (Ambion, Austin, TX, USA) that was constructed with no significant homology to sequences of any mammalian genes, served as a negative control (siRNA-neg). The appropriate transfection condition was optimized by the siRNA targeting glyceraldehyde phosphate dehydrogenase (GAPDH) (GAPDH-siRNA, Ambion, Austin, TX, USA). The transfection of siRNA was performed with the Lipofectamine 2000 (Lipo, Invitrogen, Carlsbad, CA, USA). To optimize the transfection condition, the concentration of GAPDH-siRNA varied between 10 and 100 nM. The transfection procedure was conducted according to the manufactures' instructions. Briefly, cells were passaged and reseeded in six-well plates at a density of 2×10^5 cells/well. Twenty-four hours later, GAPDH-siRNA and Lipo were diluted by Opti-MEM I (Gibco, Grand Island, NY, USA), mixed, and then added to each well. The silencing effect of GAPDH-siRNA was determined by Western blot and quantitative real-time reverse transcription polymerase chain reaction RT-PCR analyses. The optimized condition was used in subsequent experiments.

3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE ASSAY FOR CELL VIABILITY

After incubation in 96-well plates for 6 h, 24 h, 48 h, and 72 h, the cell survival was quantified by the colorimetric MTT assay. In brief, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C. Then supernatant was removed and 150 mg of dimethyl sulfoxide (DMSO) was added. Optical densities at 490 nm were measured using culture medium as a blank.

QUANTITATIVE REAL-TIME RT-PCR

After 24 h incubation, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (Takara, Kyoto, Japan) to avoid genomic DNA contamination according to the manufacturers' instructions. Samples with a 260:280 nm absorbance ratio of 1.9 or greater were reverse-transcribed using a RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, Lithuania) with random hexamer primer. PCR amplification of the cDNA template was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of amplification consisting of 95°C for 15 s and 60°C for

Table II
The base sequences of primers and probes for quantitative real-time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Taqman MGB probe (5′–3′)
HIF-1α	GAAAGAGCCCGATGCCCT	TGATATGATCGTGTCCCCAGC	FAM-ACTCTGCTAGCTCCAGC-MGB
uPA	TCACTGGCTTCGGACAAGAGA	TCCAATGTGGGACTGAATCCA	FAM-TGCTCGGAGATTCAGGAGAGG ACCTCTTA-MGB
uPAR	GTCCTGTTGGTCTTCTCCTTGTG	CACGGTGCTCGGGAATG	FAM-TCACCACCTCCAGCTCCTCGGC-MGB
PAI-1	CCTCGGTGCTGGCTATGCT	GTGCCCCTCTCACTGATATTGAA	FAM-ACCAACAGCAGGGAAAACCCGGC-MGB
tPA	GGCCAAATGCCATCAAGCT	CGTGGTATACTTCCCTGCCTTAAA	FAM-TACTGCAGAAACCCAGACCGA GACGTG-MGB
GAPDH	CAAGTTCAACGGCACAGTCAA	TGGTGAAGACGCCAGTAGACTC	FAM-TCTTCCAGGAGCGAGATCCCGCT AACT-MGB
β-actin	TCCTTCCTGGGTATGGAATC	GCACTGTGTTGGCATAGAGG	FAM-CGGATGTCAACGTCACACTTCATGA-MGB