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Identification of cathepsin B as a novel target of hypoxia-inducible factor-1-alpha in HepG2 cells

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ABSTRACT

Hypoxia-inducible factor-1-alpha (HIF-1α) activates the transcription of many genes that code for proteins involved in angiogenesis, glucose metabolism, cell proliferation/survival, and invasion/metastasis. However, the mechanisms between HIF-1 and its downstream target genes are still poorly understood. Our experimental results had shown that nuclear HIF-1α proteins were significantly induced after HepG2 cells treatment with 1% O_2 for 6 h and reached the peak expression level in 24 h. Meanwhile, the results of RT-qPCR and Western-blotting showed that HIF-1α and cathepsin B (CTSB) expressions increased with a similar pattern in response to hypoxia in the HepG2 cells. Additionally, based on bioinformatics analysis, we identified a hypoxia response element in the CTSB promoter, indicating a possible association between HIF-1α and CTSB. Moreover, luciferase assay was performed to reflect the transcriptional activity mediated through the HIF-1α binding HRE within the CTSB promoter. Furthermore, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (CHIP) revealed a specific HIF-1α binding activity to the CTSB gene promoter. For the first time, we demonstrated that CTSB is a new HIF-1α-target gene. We believe these findings will contribute to the research and development of neoplasm-targeted therapies.

1. Introduction

Hypoxia is a feature of many physiological and pathological conditions, including exposure to high altitude, fetal development in the uterus, wound healing, pulmonary fibrosis, ischemia, and neoplasia [1,2]. Hypoxia promotes tumor angiogenesis, vasculogenesis, invasiveness, and metastasis. The cellular response to decreased oxygen levels is regulated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric protein composed of HIF-1α and HIF-1β subunits [3]. Under normoxia, HIF-1α protein becomes hydroxylated at two proline residues (P402 and P564 in humans) in their oxygen-dependent degradation domain (ODDD) and is targeted by the VonHippel–Lindau (VHL) protein for proteasome-mediated degradation [4]. Under hypoxic conditions, however, HIF-1α becomes stabilized and rapidly accumulates, translocates to the nucleus, dimerizes with HIF-1β, and binds to hypoxia response elements (HREs) at the core DNA binding sequence 5′-RCGTG-3′(R, purine (A or G)) in target genes [5]. HIF-1 target genes are particularly relevant to cancer encoding angiogenic factors (VEGF, LEP, ENG, TGF-β3, LRP1), proliferation/survival factors (EPO, IGF-BP1, cyclin G2, TGF-β3, IGF2, TGF-α, WAF-1), and glucose transporters and glycolytic enzymes (PFKBF3, HK1, LDHA, ENO1, GLUT1, PFKL, GAPDH, PGK1) [3,6]. More than 2% of all genes in humans are directly or indirectly HIF-1-regulated through DNA microarray experiments [7]. The mechanisms between HIF-1 and its downstream target genes are poorly understood.

Proteases perform essential functions in the process of ovulation, fertilization, bone remodeling, cell migration, inflammation, angiogenesis, and apoptosis [8]. Cathepsin B (CTSB), a single copy gene located on chromosome 8p22, belongs to a family of

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lysosomal cysteine proteases and plays an important role in intracellular proteolysis [9]. CTSBs are found in a wide variety of human cancers including esophageal, gastric, prostate, glioblastoma, breast, colorectal carcinoma, and hepatocellular carcinoma [10]. *In vivo* models have been used to establish causal roles of CTSB in initiation, growth/tumor cell proliferation, angiogenesis, invasion, and metastasis [11]. Given the multiple roles of CTSB in tumors, a variety of CTSB inhibitors have been developed and investigated to inhibit tumor invasion in the treatment of different cancers, although clinical success has not been proven [11,12].

Identification of potential HREs in the CTSB promoter using JASPAR bioinformation database indicated that the CTSB may be directly regulated by HIF-1. To verify this, correlations among CTSB and HIF-1 expression levels were investigated in a HepG2 cell line. We further evaluated CTSB promoter activity using luciferase assays, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP).

2. Materials and methods

2.1. HepG2 cell culture and hypoxic treatment

HepG2 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM), preserved at our institute, supplemented with 100 U/mL penicillin/streptomycin and 10% fetal bovine serum (FBS). All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. For transcriptomic experiments, cells were washed and exposed to hypoxia (1% O_2) for 24 h in a serum-free DMEM medium (SFM). After incubation, cells were harvested and used for total RNA extraction. For proteomic experiments, after treatment with hypoxia (1% O_2) for 24 h, the media were collected and the supernatant proteins prepared with TCA/acetone precipitation according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK) after concentration using Amicon Ultra Centrifugal Filter Devices (Millipore, New York, USA). The cells were washed with cold phosphate-buffered saline (PBS), harvested with a cell scraper, and directly processed for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

2.2. Nuclear extract preparation and Western blot analysis of HIF-1α

The HepG2 cells were incubated for 6, 12, 24, or 48 h under hypoxic conditions (1% O_2) or for 24 h under normoxia and resuspended in 200 μL of hypotonic lysis buffer: 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 20 min. Then, 10 μL 10% NP-40 was added and cells vigorously mixed and centrifuged for 1 min. The nuclei pellets were suspended in 50 μL of buffer containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol (v/v); mixed for 20 min; and centrifuged to produce supernatant containing nuclear proteins. The proteins determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) were resolved in sodium dodecyl sulfate-PAGE (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blockage in tris-buffered saline (TBS) containing 5% nonfat milk, membranes were probed with anti-HIF-1α monoclonal antibody (BD Transduction, San Jose, CA, USA) in TBS with 5% nonfat milk. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G for monoclonal primary antibody combined with ECL Chemiluminescent Substrate (Amersham Biosciences, Little Chalfont, UK) was used for signal detection.

2.3. Western blot analysis and real-time polymerase chain reaction (qPCR)

Western blot analysis and real-time polymerase chain reaction was performed as described previously [13]. Antibodies directed against CTSB and β-actin were obtained from Sigma (St. Louis, MO, USA). Total protein extracts were prepared from HepG2 cells treated under hypoxic conditions (1% O_2) for 6, 12, 24, and 48 h. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The following primer sets were used for real-time polymerase chain reaction (qPCR): CTSB, 5′-GGCCTCTATGACTCGCATGT-3′ and 5′-TTTGTAGGACGGGGTGTAGC-3′ and β-actin, 5′- GCGACTTGACC-GACTACCT-3′ and 5′- CCGCACGATTCCATACCC -3′.

2.4. Luciferase assays

Luciferase assay was performed as described previously [13]. Various fragments (P1, nucleotide, nt -1874 to -828; P2, nt -1342 to -828; P3, nt -1052 to -828; P4, nt -912 to -526) of the human CTSB promoter were amplified and subcloned into the pGL4.17 (Promega). According to the manufacturer's instructions, site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

2.5. EMSA and ChIP

EMSA and ChIP assays was performed as described previously [13]. The corresponding to the complementary oligonucleotides sequence spanning nt −1201 to −1178 in the CTSB promoter (5′-CAGGCGTGAGCCACTGCACCCGGC-3′ and 5′- GTCCGCACTCGGTGAC GTGGGCCG-3′) or containing a mutated HIF-1α response element (5′-CAGACGTTAGCCACTGTACACGGC-3′ and 5′- GTCTGCAATCGGT-GACATGTGCCG-3′), were labelled with biotin. EMSA was carried out using the LightShift® Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA). Using a nuclear protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China), the nuclear proteins were extracted from HepG2 cells treated under hypoxic conditions (1% O_2) for 24 h.

ChIP assays were performed using the Magna ChIP™ G kit (Upstate Biotechnology, Inc., Billerica, MA, USA) according to the manufacturer's protocol. Immunoprecipitation was performed using HIF-1α-specific antibodies (Cell Signaling Technology, Danvers, MA, USA). After ChIP, DNA was purified using the Qiagen Lambda Midi Kit (Hilden, Germany). Input DNA (1/100 of total immunoprecipitated chromatin) and immunoprecipitated DNA were subjected to PCR analysis using primers specific for the CTSB promoter (5′-TCTTGATCTCCTGACCTGTGA-3′ and 5′-CTGTGGCGACAGTAAGAG G-3′, 105-bp product), and the VEGF promoter (5′-AGACTCCA-CAGTGCATACGTG-3′ and 5′-TTGTGGAGCTGAGAACGGGA-3′, 98-bp), as indicated.

2.6. Statistical analysis

All data are displayed as the mean ± SD. The statistical difference between the three groups was analyzed by the Student-Newman-Keuls test for multiple comparisons. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Low oxygen upregulates nuclear HIF-1α protein levels in $HepG_2$ cells

We investigated whether HIF-1α is induced under a hypoxic

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