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Hypoxia induces peroxisome proliferator-activated receptor γ expression via HIF-1-dependent mechanisms in HepG2 cell line

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) can activate expression of a broad range of genes in response to hypoxia. It has been shown that the levels of peroxisome proliferator-activated receptor γ (PPARγ) are influenced by changes in oxygen tension, and PPARγ plays a critical role in metabolism regulation and cancers. In this research, we observed an increased PPARγ mRNA and protein levels in company with increased HIF-1 protein levels in HepG2 cells in hypoxia as compared with in normoxia. Enforced expression of *HIF-1α* induced PPARγ1 and PPARγ2 expression, while knockdown of *HIF-1α* by small interference RNA deduced PPARγ1 and PPARγ2 expression in HepG2 cells under hypoxic conditions. By dual-luciferase reporter assay and chromatin immunoprecipitation assay we confirmed a functional hypoxic response element (HRE) localized at 684 bp upstream of the transcriptional start site (TSS) of *PPARγ1* and a functional HRE localized at 204 bp downstream of the TSS of *PPARγ2* in HepG2 cells. Additionally we observed an increase and co-presence of PPARγ and HIF-1α, and a highly positive correlation between PPARγ expression and HIF-1α expression ($r = 0.553$, $p < 0.0001$), in the same tumor tissue areas of hepatocellular carcinoma patients. Our data suggested a new mechanism of hepatocellular carcinoma cells response to hypoxia.

Introduction

Hypoxia is a feature of most tumors. It promotes tumor angiogenesis, vasculogenesis, invasiveness and metastasis. Hypoxia-inducible factors (HIFs)[2] play a central role in cellular adaptation to hypoxia [1]. Over-expression of HIF-1α, the regulatory subunit of HIF-1, is associated with increased glycose metabolism [2,3], lipid accumulation [4], vascular density and tumor growth [5]. Functional HIF-1 exists as an α/β-heterodimer, the activation of which is dependent upon stabilization of an O_2-dependent degradation domain of the α-subunit by the ubiquitin–proteasome pathway [6]. Under normoxia, HIF-1α protein becomes hydroxylated at proline 564 in its O_2-dependent degradation domain [7] and is targeted by the Von Hippel-Lindau (VHL) protein for proteosome-mediated degradation [8]. Under hypoxic conditions, however, HIF-1α becomes stabilized and rapidly accumulates, translocates to the nucleus and dimerizes with HIF-1β, binds to hypoxia response elements (HRE), a core DNA binding sequence 5′-RCGTG-3′ (R, purine (A or G)) in target genes [9,10]. Then HIF-1 recruits co-activators and activates transcription of a number of genes including vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF1) and lysyl oxidase (LOX) [5], which can stimulate angiogenesis, cell survival, and metastasis. Some studies reported that the PPARγ levels are influenced by changes in oxygen tension [11–16].

PPARγ is a ligand-dependent nuclear transcription factor. Four major transcriptional start sits (TSS) of human *PPARγ* have been identified by differential promoter usage, and four mature mRNA transcripts are generated through different initiation and alternative splicing at the 5′-terminal region [17]. PPARγ1, γ3, and γ4 mRNA transcripts translate into the identical PPARγ1 protein, while PPARγ2 mRNA transcript produces PPARγ2 protein with 30 extra N-terminal amino acids. PPARγ1 is expressed in all PPARγ-expressing tissues and cells whereas PPARγ2 is almost exclusively found in adipose tissue, where it exerts a pronounced adipogenic activity [18]. It is well known that PPARγ is not only involved in the regulation of glucose and lipid metabolism, but also play a critical role in the development of cancers [19,20]. Recent works

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[2] *Abbreviations used:* HIF-1, hypoxia-inducible factor-1; PPARγ, peroxisome proliferator-activated receptor γ; DFO, desferrioxamine; HRE, hypoxia response element; ChIP, chromatin immunoprecipitation; TSS, transcriptional start site; siRNA, small interference RNA; VEGF, vascular endothelial growth factor.

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revealed high levels of PPARγ expression in some cancers, including colon, breasts, prostate, and hepatocellular carcinoma [21–25]. Some studies reported that PPARγ mRNA increased in fetal sheep adipose tissue in response to long-term hypoxia [13] and in differentiation of C2C12 and G8 cell lines into adipocytes in hypoxia [12]. It is reported that peroxisome proliferator-activated receptor γ (PPARγ) gene is a direct transcriptional target of HIF-1 in mouse cardiomyocytes [26]. However, the role of HIF-1 in regulating the expression of PPARγ in human cancer remained unclear.

In the present study we attempted to look for a link between hypoxia and PPARγ expression in cancer. We demonstrated that hypoxia up-regulates the expression of PPARγ1 and PPARγ2 in a HIF-1-dependent manner in HepG2, a human hepatocellular carcinoma cell line. In addition, we observed an increase and co-presence of PPARγ and HIF-1α in the same areas of hepatocellular carcinoma tissues. These results suggested a molecular mechanism that HIF-1 mediates cellular responses to hypoxia in hepatocellular carcinoma cells.

Materials and methods

Cell culture and hypoxic exposures

Human hepatocellular carcinoma cell line HepG2 was obtained from Cell Culture Center of Peking Union Medical College. HepG2 cells were grown in modified Eagle's medium (MEM) (Gibco, Grand Island, NY) supplemented with 10% FBS, and Non-essential amino acids (Gibco, Grand Island, NY) at 37 °C in an incubator with a controlled humidified atmosphere containing 5% CO_2. For hypoxic stimulation, cells were placed in an incubator chamber that was tightly sealed and thoroughly flushed with a gas mixture containing 5% CO_2, 1% O_2 and 94% nitrogen at 37 °C. For a mimetic hypoxia desferrioxamine (DFO) (Sigma–Aldrich, Deisenhofen) was added to the medium at a final concentration of 100 μM.

Cell transfection

For transfection with plasmids, HepG2 cells were planted in 6-well plates at 50–70% confluency and cultured for 24 h before transfected with 2 μg of plasmid. The transfection reagent Lipofectamine LTX and Plus (Invitrogen, Carlsbad, CA) were used according to the manufacturer's instructions. For transfection with siRNAs, HepG2 cells were planted in 6-well plates at 50–70% confluency and cultured for 24 h. The siRNAs were resuspended and DharmaFECT transfection reagents (Dharmacon, Lafayette, CO) were used according to the manufacturer's instructions. The siRNAs targeting HIF-1α were siGENOME SMARTpool (Thermo Fisher Scientific, Waltham, MA) containing GGACACAGAUUUAGACUUG, GAUGGAAGCACUAGACAAA, CGUGUUAUCUGUCGCUUUG, and CAUGAAAGA AUUACCGAAU.

RNA isolation and real-time PCR analysis

Cells were dissolved in TRIZOL reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted according to the manufacturer's instructions. Total RNA was converted into cDNAs using the M-MLV reverse-transcriptional system (Invitrogen, Carlsbad, CA) in the presence of random primer. The cDNAs were used for real-time PCR with specific primers pairs (Supplementary Table S1). SYBR green PCR mix (Transgen, Beijing) was used for the real-time PCR. The relative abundance of PPARγ or PPARγ2 transcript was quantified using the comparative Ct method with β-actin as an internal control.

Western blotting

Cell lysates were subjected to SDS–PAGE and transferred to a PVDF membrane. Membranes were blocked with a 5% nonfat dry milk solution and incubated with either anti-HIF-1α (ab-2185, Abcam, Cambridge, MA), anti-PPARγ (sc-7273, Santa cruz Biotechnology) or anti-β-actin (Proteintech, Chicago, IL) overnight at 4 °C followed by peroxidase-conjugated affinipure goat anit-mouse IgG (H+L) (Zhongshan Goldenbridge, Beijing). After washing with TBS-T buffer, the membrane was treated with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica) and exposed to Kodak X-omat BT Film.

Plasmid constructs

The HIF-1α cDNA sequence has been successfully constructed in the pcDNA6V5HisB plasmid in our laboratory [27].

The −1853∼−171 region of *PPARγ1* promoter (*PPARγ1*HRE1′-5′), the −2926∼−1470 region (*PPARγ2* HRE2∼4) and the −411∼+392 region (*PPARγ2* HRE0∼1) of *PPARγ2* promoter were amplified from human genomic DNA by PCR and inserted into the luciferase reporter vector pGL3-Basic (Promega, Madison, WI). The putative HREs core sequence (5′-RCGTG-3′) were deleted, respectively by PCR amplification. The deletion mutants were then inserted into pGL3-basic, respectively. The PCR primers were shown in Supplementary Table S1. The nucleotide sequence containing the identified HRE of *EPO* gene [28] was also cloned into pGL3-promoter, as a positive control.

Dual-luciferase reporter assay

HepG2 cells were plated into 24-well plates to reach approximately 50–70% confluence on the next day. The cells were co-transfected with pGL3-basic-based construct and pRL-TK plasmid DNAs using Lipofectamine LTX and Plus. The transfection medium was replaced with complete medium after 6 h. The cells were incubated under normoxic and hypoxic conditions for an additional 18 h. Then cells were lysed with Passive Lysis Buffer and reporter gene expression was assessed using the dual-luciferase reporter assay system (Promega, Madison, WI).

Chromatin immunoprecipitation (ChIP)

HepG2 cells were plated into 100 mm dishes and grown to about 70% confluence. Then the cells were exposed in normoxia or hypoxia for 24 h and then fixed in 1% formaldehyde (Sigma–Aldrich, St. Louis, Missouri) at room temperature for 10 min, and quenched with glycine for 5 min. Cell were lysed and sonicated to get 200–1000 bp DNA fragments. Chromatin immunoprecipitation was performed using the EZ-ChIP™ Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA) according to the manufacturer's instructions with minor modifications. A ChIP-grade rabbit polyclonal antibody to HIF-1α (ab-2185, Abcam, Cambridge, MA) was used as the immunoprecipitating antibody and rabbit IgG (ab2410, Abcam, Cambridge, MA) was used as the control. After reverse cross-linking and DNA purification, the INPUT and the immunoprecipitated DNA samples were used as templates to amplify the target sequences by PCR and real-time PCR. The primers for amplifying the DNA regions containing the potential HREs within the *PPARγ1* promoter and the *PPARγ2* gene by ChIP PCR and ChIP real-time PCR were shown in Supplementary Table S1. The PCR products were assayed by agarose gel electrophoresis. ChIP real-time PCR results were analyzed by evaluating signal of enrichment over noise normalized to the input DNA. A 10-fold serial dilutions of the Input Control DNA (1:1–1:1000) were adopted for determining the amplification efficiency. An