Hypoxic repression of CYP7A1 through a HIF-1α- and SHP-independent mechanism

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Liver cells experience hypoxic stress when drug-metabolizing enzymes excessively consume O2 for hydroxylation. Hypoxic stress changes the transcription of several genes by activating a heterodimeric transcription factor called hypoxia-inducible factor-1α/β (HIF-1α/β). We found that hypoxic stress (0.1% O2) increased the expression of cytochrome P450 7A1 (CYP7A1), a rate-limiting enzyme involved in bile acid biosynthesis. Chenodeoxycholic acid (CDCA), a major component of bile acids, represses CYP7A1 by activating a transcriptional repressor named small heterodimer partner (SHP). We observed that hypoxia decreased the levels of both CDCA and SHP, suggesting that hypoxia repressed CYP7A1 without inducing SHP. The finding that overexpression of HIF-1α increased the activity of the CYP7A1 promoter suggested that hypoxia decreased the expression of CYP7A1 in a HIF-1α-independent manner. Thus, the results of this study suggested that hypoxia decreased the activity of CYP7A1 by limiting its substrate O2, and by decreasing the transcription of CYP7A1. [BMB Reports 2016; 49(3): 173-178]

INTRODUCTION

Partial O2 pressure in the liver ranges from 2% in the perivenous area to 6% in the perportal area. O2 concentration in the liver tissue decreases with an increase in O2 consumption and decrease in O2 supply (1, 2). The liver contains many drug-metabolizing enzymes such as cytochrome P450 isofoms. Most of these enzymes use O2 as a substrate for hydroxylation. Expression and catalytic activities of these enzymes are regulated by several signals such as redox status, xenobiotics, and hormones, suggesting that O2 consumption in the liver is regulated by these signals. For example, both chronic and acute alcohol uptake increases O2 consumption in the liver by inducing various hydroxylation enzymes, including CYP2E1, leading to severe local hypoxia in the liver tissues (3).

Hypoxia increases the transcription of several genes involved in glycolysis, angiogenesis, metastasis, and fibrosis. Most hypoxia-induced genes are directly activated by a common transcription factor named hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer composed of α and β subunits. Under normoxia, HIF-1α-specific prolyl-4-hydroxylase hydroxylates HIF-1α by using molecular O2 and α-ketoglutarate. Hydroxylated HIF-1α is recognized by an E3 ubiquitin ligase called von Hippel-Lindau protein. Finally, ubiquitinylated HIF-1α is degraded by 26S proteasome. Hypoxia prevents the hydroxylation of HIF-1α and stabilizes it. HIF-1α forms a heterodimer with HIF-1β/ARNT and binds to hypoxia-responsive elements (HREs) in its target genes such as genes encoding erythropoietin, BCL2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), carbonic anhydrase 9 (CA9), and vascular endothelial growth factors (4-8).

In the liver, bile acids are synthesized by the sequential hydroxylation of cholesterol. Bile acid synthesis is regulated by an interplay among various steroid receptors. Bile acid decreases the expression of cytochrome P450 7A1 (CYP7A1), which catalyzes 7α-hydroxylation of cholesterol. This first hydroxylation of cholesterol is a rate-limiting step which is regulated by bile acid derivatives and hormones through a negative feedback mechanism (9). The promoter region of CYP7A1 contains several regulatory elements such as hepatocyte nuclear factor 4α (HNF-4α) binding site, liver receptor homolog-1 (LRH-1) responsive element, and liver X receptor (LXR) response element (LXRE). Oxycholesterol binds to LXRα, and the liganded LXRα forms a heterodimer with retinoid X receptor (RXR) and interacts with LXRE in the CYP7A1 promoter, thus increasing the transcription of CYP7A1 (10-12). Upregulation of CYP7A1 increases bile acid synthesis. Chenodeoxycholic acid (CDCA), a major component of bile acids, is an endogenous ligand of farnesoid X receptor (FXR). CDCA-bound FXR induces the expression of small heterodimer partner (SHP), a transcriptional repressor. SHP then interacts with the transactivator LRH-1 and prevents it from activating its target genes CYP7A1 and SHP. Thus, SHP represses the expression of CYP7A1 and the gene coding itself.

In this study, we investigated whether hypoxic stress in liver cells influenced bile acid synthesis. We observed that hypoxia
repressed CYP7A1 and decreased CDCA levels. These findings suggested that hypoxia in the liver decreased bile acid synthesis by repressing CYP7A1.

RESULTS AND DISCUSSION

Hypoxia represses CYP7A1 in a HIF-1-independent manner

We observed that severe hypoxia (0.1% O2) induced phosphoglycerate kinase-1 (PGK-1) and carbonic anhydrase 9 (CA9), hypoxic target genes, but repressed SHP mRNA expression (Fig. 1A-1C). We examined whether severe hypoxia altered CYP7A1 expression, which is repressed by SHP. We observed that severe hypoxia repressed CYP7A1; however, levels of its repressor SHP were also reduced (Fig. 1D and 1E).

The promoter region of human CYP7A1 has been studied intensively. This region has binding sites for HNF-4, LRH-1, and LXR, which activate the transcription of CYP7A1. To determine whether HIF-1α was involved in the hypoxic repression of CYP7A1, we transfected HepG2 cells with a reporter plasmid encoding a luciferase gene under the control of the CYP7A1 promoter and exposed these transfected cells to CDCA or hypoxic stress. We observed that both CDCA and hypoxic stress decreased the activity of the CYP7A1 promoter (Fig. 2B). Next, we transfected HepG2 cells with the reporter plasmid and cDNA encoding HIF-1α and observed that overexpression of HIF-1α increased the expression of the reporter gene under the control of HREs. Interestingly, we observed that overexpression of HIF-1α increased the activity of the human CYP7A1 promoter (Fig. 2C and 2D). These results suggested that hypoxia decreased the activity of the CYP7A1 promoter in a HIF-1α-independent manner. Because HIF-1α functions as a transactivator, it can be suggested that hypoxia repressed CYP7A1 through inhibitory pathways that could override the positive effects of the HIF-1α/β heterodimer.

Hypoxia-associated changes in CDCA and SHP levels in HepG2 cells

Because CDCA is a major final product of CYP7A1 and because hypoxia repressed the expression of CYP7A1, we examined whether hypoxia decreased the levels of CDCA. Results of HPLC showed that hypoxia decreased the levels of CDCA (Fig. 3A). CDCA is an endogenous agonist of FXR. Thus, our result suggested that hypoxia decreased the expression of SHP, an FXR target gene, at least in part by decreasing CDCA levels.
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Fig. 2. Human CYP7A1 promoter. (A) A schematic diagram of the human CYP7A1 promoter showing bile acid-responsive elements (BAREs) and E box sequence (CACGTG). Arrows indicate E-boxes located on the sense or antisense strands of CYP7A1 promoter. (B) Reporter analyses. HepG2 cells were transfected with 1 μg of the indicated reporter plasmids (h1887-Luc plasmid, a human CYP7A1 promoter [-1887/+24]-Luc reporter plasmid containing upstream region from -1887 to +24, and h371-Luc plasmid, a human CYP7A1 promoter [-371/+24]-Luc plasmid containing upstream region from -371 to +24, and 100 ng of CHO10 plasmid. The cells were serum starved in a medium supplemented with 0.5% FBS for 20 hours and were treated with CDCA or 0.1% O2 for 24 hours; RLU, relative luciferase units. (C) Analysis of luciferase activity. HepG2 cells were transfected with 500 ng of h371-Luc plasmid and 1 μg of HIF-1α-encoding pCMV-HIF-1α plasmid or an empty pCMV plasmid. (D) Analysis of luciferase activity. HepG2 cells were transfected with 100 ng of pHRE-Luc plasmid and 1 μg of HIF-1α-encoding pCMV-HIF-1α plasmid or an empty pCMV plasmid. The cells were serum starved in a medium supplemented with 0.5% FBS for 20 hours and were treated with 0.1% O2 for 24 hours. *P \leq 0.05.

Fig. 3. Levels of CDCA, SHP, and CYP7A1 in hypoxic HepG2 cells. (A) HPLC of CDCA secreted in hypoxic media. Media in which HepG2 cells were incubated under normoxia or in 0.1% O2 for 16 hours were collected. (B and C) Quantitative RT-PCR of SHP and CYP7A1. Serum-starved HepG2 cells were pretreated with 100 μM CDCA for 6 hours and were exposed to 20%, 5%, or 0.1% O2 for 32 hours. *P \leq 0.05; **P \leq 0.1.
Consistent with this idea, we observed that CDCA treatment recovered SHP expression even under severe hypoxia (Fig. 3B). CDCA, an SHP inducer, dramatically decreased the expression of CYP7A1, and severe hypoxia maintained the repression of CYP7A1 even after CDCA treatment (Fig. 3C). The finding that hypoxia decreased the expression of both CYP7A1 and SHP suggested that hypoxia repressed CYP7A1 through a mechanism that was independent of FXR and SHP.

The results of this study indicated that hypoxic stress decreased the expression of CYP7A1 and its canonical repressor SHP, suggesting that hypoxia repressed CYP7A1 in an SHP-independent manner. Studies on Shp-null mice indicated that both SHP-dependent and SHP-independent negative regulatory mechanisms control the expression of Cyp7a1. Lithocholic acid, a potent cholestatic agent, activates nuclear pregnane X receptor (PXR), and the liganded PXR represses Cyp7a1 even in Shp-null mice (13,14). However, it is unclear whether hypoxic repression of CYP7A1 is mediated by PXR.

Alternatively, Noshiro et al. showed that Cyp7a1 is repressed by differentially embryo chondrocyte 2 (DEC2) (15). This and our previous finding that both DEC1 and DEC2 are induced by the results of this study showed that hypoxia repressed the expression of CYP7A1 (20-23).

Oxygen gradient is a key regulator of the expression and activity of metabolic enzymes in the liver parenchyma. Intracellular pO2 levels in the liver were approximately 45-50 mmHg in the periportal and 15-20 mmHg in the perivenous area. Bile concentration dropped by about six-folds from the periportal to the perivenous area, implying that the rate of bile synthesis is positively correlated with pO2 levels (2). A recent study demonstrated that hepatic and systemic cholesterol levels are increased with a concurrent decrease in hepatic bile acid synthesis (19). Because drug-metabolizing cytochrome P450 enzymes use O2 for hydroxylation, an increase in drug metabolism induces hypoxic stress, thus decreasing bile acid synthesis (19). Because drug-metabolizing cytochrome P450 enzymes use O2 for hydroxylation, an increase in drug metabolism induces hypoxic stress, thus decreasing bile acid synthesis.

CYP7A1 is positively correlated with pO2 levels (2). A recent study demonstrated that hepatic and systemic cholesterol levels are increased with a concurrent decrease in hepatic bile acid synthesis (19). Because drug-metabolizing cytochrome P450 enzymes use O2 for hydroxylation, an increase in drug metabolism induces hypoxic stress, thus decreasing bile acid synthesis.

Northern analyses and quantitative real-time reverse transcription-polymerase chain reaction
Northern analyses and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) were performed as described previously (24). Expression level of 18S rRNA was used for normalization. The following forward and reverse primers were used for qRT-PCR: human CYP7A1, 5'-CACATGCCCCGGAGAAA-3' and 5'-AAAGTCGCTGGAA-TGGTGTGG-3' (GenBank accession number: M93133); human SHP, 5'-AGATGTTGACATGCGTGGCCCTCTT-3' and 5'-AGAGGTCTTCTCCAAGGACCG-3' (GenBank accession number: NM_021969); human HIF-1α, 5'-CCTTCGCTCTGG-3' and 5'-GCAGCTAGGAATAATGGAATA-3' (GenBank accession number: NM_001216.2); and 18S rRNA, 5'-ACC-GAAGCTAGACACATGTG-3' and 5'-TGGATCCGAGCG-3' (GenBank accession number: NM_001216.2); and 18S rRNA, 5'-ACC-GAAGCTAGACACATGTG-3' and 5'-TGGATCCGAGCG-3' (GenBank accession number: NM_001216.2). Consistently with our in vitro results, in both mice models, Cyp7a1 and other genes involved in bile acid synthesis were repressed (18).

Our finding that hypoxia decreased the expression of CYP7A1 and the fact that CYP7A1 enzyme uses O2 for its catalytic reaction indicated that hypoxic stress in the liver decreased both the amount and activity of CYP7A1, thus decreasing bile acid synthesis (19). Because drug-metabolizing cytochrome P450 enzymes use O2 for hydroxylation, an increase in drug metabolism induces hypoxic stress, thus decreasing bile acid synthesis. Hypoxia increases the transcription of other hydroxylases such as HIF-1α-specific prolyl-4-hydroxylases, collagen prolyl-4-hydroxylase, and histone demethylases, thus compensating their decreased catalytic activities. However, the results of this study showed that hypoxia repressed the expression of CYP7A1 (20-23).

MATERIALS AND METHODS

Materials
CDCA was purchased from Sigma Aldrich (St. Louis, MO, USA). A cDNA encoding human HIF-1α (U22431) was inserted in expression vectors for transfection assays. In addition, we used pHRE-Luc reporter plasmid containing 4 copies of erythropoietin HRE (nucleotides 3449-3470) (24). Human CYP7A1 promoter-Luc reporter plasmids were provided by Dr J. K. Kemper (25).

Cells and treatments
Human HepG2 cells were purchased from American Type Culture Collection (Manassas, VA, USA) (21). HepG2 cells were cultured at 37°C in MEM supplemented with non-essential amino acids and 10% FBS in humidified air containing 5% CO2. Before CDCA treatment, HepG2 cells were serum starved in a medium supplemented with 0.5% FBS for 20 hours. The cells were pretreated with CDCA for 6 hours and were exposed to hypoxic stress (0.1% or 5% O2) by incubating them in an anaerobic incubator (Model 1029; Forma Scientific, Inc., Marietta, OH, USA) or in an Invivo200 Hypoxia Workstation (Ruskin Technology, Leeds, UK).

Transient transfection and luciferase assay
HepG2 cells were plated at a density of 1.0 × 105 cells/well in a 12-well plate. After 18 hours, transfection was performed using Lipofectamine LTX and PLUS reagent (Invitrogen, Carlsbad, CA, USA). Before treatment, the cells were serum starved in a medium supplemented with 0.5% FBS for 20-24 hours. Cell extracts were prepared and were analyzed using a luciferase assay system (Promega, Madison, WI, USA). Luciferase activity was normalized using β-galactosidase activity, total protein concentration, or Renilla luciferase activity.
Measurement of CDCA levels by performing high-performance liquid chromatography

HepG2 cells were seeded in a 60-mm tissue culture plate and were grown to 70% confluency for 20 hours. The medium was replaced by 6 ml serum-free MEM. The cells were then serum starved for 20 hours before exposure to hypoxia for 8 or 16 hours. Cell-free media were used for performing high-performance liquid chromatography (HPLC). Standard stock solution of CDCA (50 μM) was prepared using DMSO. The solution was diluted with methanol to obtain working standard solutions of different concentrations. Calibration curves were obtained using 12 calibration standards corresponding to different final concentrations of CDCA (0.3, 0.6, 1.5, 3, 6, 15, 30, 60, 150, 300, 600, and 1,500 pmol).

HPLC was performed using LC-10ADvp binary pump system, SIL-10ADvp autosampler, and CTO-10Avp oven (Shimadzu, Kyoto, Japan). Analytical column used was Capcell Pak phenyl column (150 x 1.5 mm ID, 5 μm; Shiseido, Japan). Mobile phases consisted of 5 mM ammonium formate buffer (pH 4.0, buffer A) and 5 mM ammonium formate in 90% methanol (buffer B). A gradient program was used for HPLC separation, with a flow rate of 0.15 ml/min. The initial composition of buffer B was 50%. This was increased to 65% after 1 min and then to 90% after 15 min, which was maintained for 0.2 min. Finally, the composition was re-equilibrated to the initial composition for 0.01 min. Each run time was 17 min. The HPLC system was as described previously (24). CDCA was analyzed by monitoring their parent ions at m/z 391.

Statistical analysis

The P value was analyzed using 2-tailed Student’s t-test. All error bars represent standard error of mean (SEM). The experiments were repeated 2-3 times. Therefore, 6-9 values of qPCR were used for statistical analyses.

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