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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 O_2 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% O_2) decreased 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-1-independent manner. Thus, the results of this study suggested that hypoxia decreased the activity of CYP7A1 by limiting its substrate O_2, and by decreasing the transcription of *CYP7A1*. [BMB Reports 2016; 49(3): 173-178]

INTRODUCTION

Partial O_2 pressure in the liver ranges from 2% in the perivenous area to 6% in the periportal area. O_2 concentration in the liver tissue decreases with an increase in O_2 consumption and decrease in O_2 supply (1, 2). The liver contains many drug-metabolizing enzymes such as cytochrome P450 isoforms. Most of these enzymes use O_2 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 O_2 consumption in the liver is regulated by these signals. For example, both chronic and acute alcohol uptake increases O_2 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 O_2 and α-ketoglutarate. Hydroxylated HIF-1α is recognized by an E3 ubiquitin ligase called von Hippel–Lindau protein. Finally, ubiquitinated 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

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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% O_2) 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.

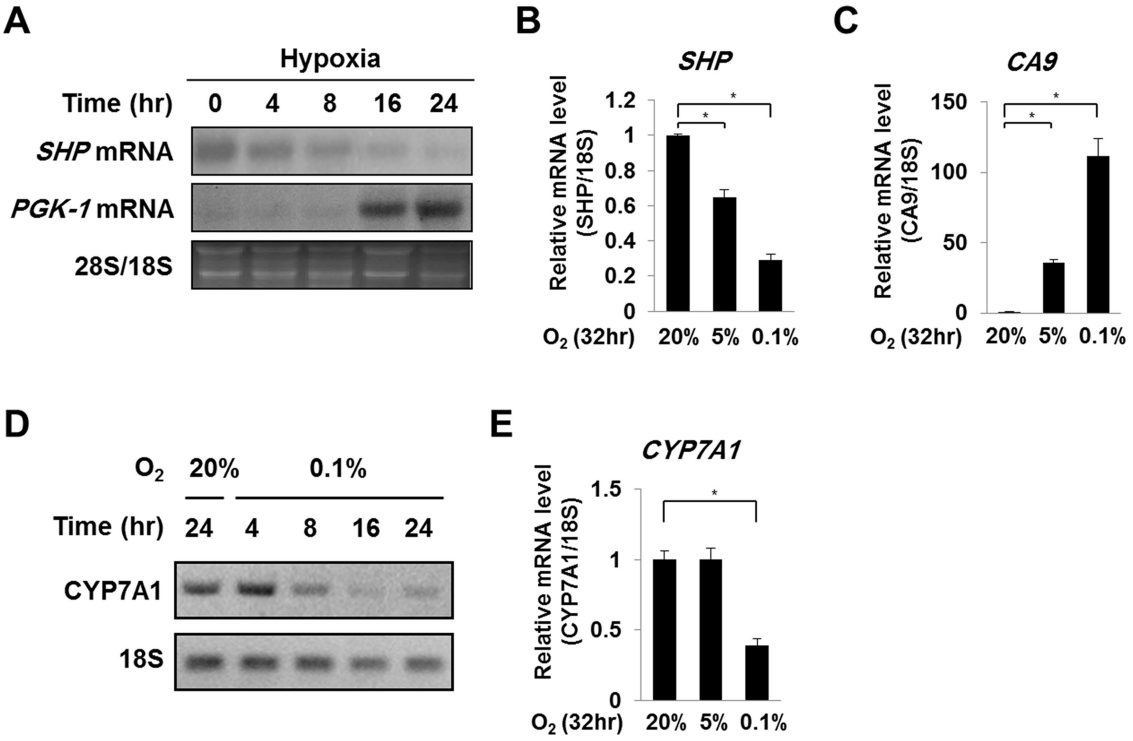

Fig. 1. Expression of *SHP* and *CYP7A1*. (A) Northern analyses. HepG2 cells were serum starved in a medium supplemented with 0.5% FBS for 20 hours before exposure to 0.1% O_2 for the indicated times. ^{32}P-labeled cDNAs were used as indicated above. *SHP* and *PGK-1* mRNA levels were determined by exposure to an X-ray film. Densities of 18S and 28S rRNAs are shown. (B and C) Quantitative RT-PCR of *SHP* and *CA9*. After serum starvation, HepG2 cells were exposed to 20%, 5%, or 0.1% O_2 for 32 hours. (D) RT-PCR of *CYP7A1*. 18S rRNA was used as a loading control. (E) Quantitative RT-PCR analyses of *CYP7A1*. *P $\leq$ 0.05.

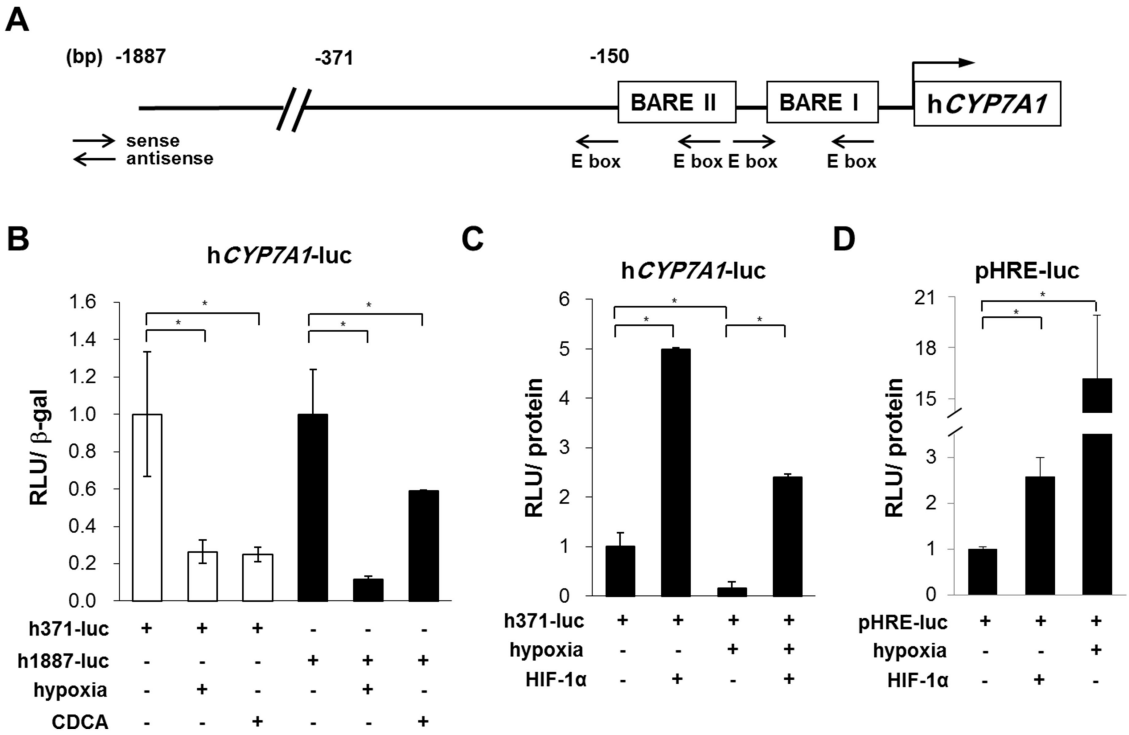

Fig. 2. Human *CYP7A1* promoter. (A) A schematic diagram of the human *CYP7A1* promoter showing bile acid-responsive elements (BAREs) and E box sequence (CACGTG). Arrows indicate E-boxes located on the sense or antisense strands of *CYP7A1* promoter. (B) Reporter analyses. HepG2 cells were transfected 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% O_2 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% O_2 for 24 hours. *P $\leq$ 0.05.

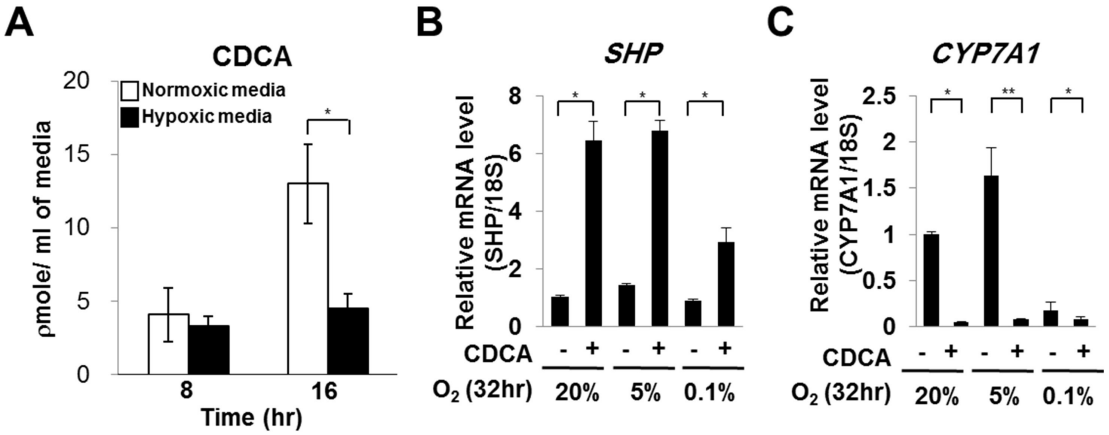

Fig. 3. Levels of CDCA, *SHP*, and *CYP7A1* in hypoxic HepG2 cells. (A) HPLC of CDCA secreted in hypoxic media. Media in which HepG2 cells were incubated under normoxia or in 0.1% O_2 for 16 hours were collected. (B and C) Quantitative RT-PCR of *SHP* and *CYP7A1*. Serum-starved HepG2 cells were pretreated with 100 μM CDCA for 6 hours and were exposed to 20%, 5%, or 0.1% O_2 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 differentiated embryo chondrocyte 2 (DEC2) (15). This and our previous finding that both DEC1 and DEC2 are induced by HIF suggest that hypoxia-induced DEC2 mediates the hypoxic repression of *Cyp7a1* (16, 17). However, our finding that cotransfection of gene encoding HIF-1α increased the activity of the *CYP7A1* promoter suggested that HIF-1 activation did not induce the hypoxic repression of *CYP7A1*.

Oxygen gradient is a key regulator of the expression and activity of metabolic enzymes in the liver parenchyma. Intracellular pO_2 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 pO_2 levels (2). A recent study demonstrated that hepatic and systemic cholesterol levels are increased with a concurrent decrease in hepatic bile acid synthesis in two hypoxic mouse models, an acute lung injury model and mice exposed to 10 % O_2 for 3 weeks. 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 O_2 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 O_2 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% CO_2. 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% O_2) 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).

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′-CACAATGCCCGGGAGAAA-3′ and 5′-AAAGTCGCTGGAATGGTGTTTG-3′ (GenBank accession number: M93133); human *SHP*, 5′-AGATGTTGACATCGCTGGCCTTCT-3′ and 5′-AGAGCTGTTCCTAAGGAGCCAAGT-3′ (GenBank accession number: NM_021969); human *CA9*, 5′-CAGTTGCTGTCTCGCTTGGA-3′ and 5′-TGAAGTCAGAGGGCAGGAGTG-3′ (GenBank accession number: NM_001216.2); and 18S rRNA, 5′-ACCGCAGCTAGGAATAATGGAATA-3′ and 5′-CTTTCGCTCTGGTCCGTCTT-3′ (GenBank accession number: X03205).

Transient transfection and luciferase assay

HepG2 cells were plated at a density of 1.0×10^5 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-10AD*vp* autosampler, and CTO-10A*vp* oven (Shimadzu, Kyoto, Japan). Analytical column used was Capcell Pak phenyl column (150 × 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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REFERENCES

1. Nath B and Szabo G (2012) Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. Hepatology 55, 622-633
2. Jungermann K and Kietzmann T (2000) Oxygen: modulator of metabolic zonation and disease of the liver. Hepatology 31, 255-260
3. Arteel GE, Iimuro Y, Yin M, Raleigh JA and Thurman RG (1997) Chronic enteral ethanol treatment causes hypoxia in rat liver tissue in vivo. Hepatology 25, 920-926
4. Masson N and Ratcliffe PJ (2003) HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O(2) levels. J Cell Sci 116, 3041-3049
5. Bruick RK and McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294, 1337-1340
6. Jaakkola P, Mole DR, Tian YM et al (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292, 468-472
7. Kallio PJ, Wilson WJ, O'Brien S, Makino Y and Poellinger L (1999) Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J Biol Chem 274, 6519-6525
8. Park KC, Lee DC and Yeom YI (2015) NDRG3-mediated lactate signaling in hypoxia. BMB Rep 48, 301-302
9. Kim I, Ahn SH, Inagaki T et al (2007) Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. J Lipid Res 48, 2664-2672
10. Davis RA, Miyake JH, Hui TY and Spann NJ (2002) Regulation of cholesterol-7alpha-hydroxylase: BAREly missing a SHP. J Lipid Res 43, 533-543
11. Bavner A, Sanyal S, Gustafsson JA and Treuter E (2005) Transcriptional corepression by SHP: molecular mechanisms and physiological consequences. Trends Endocrinol Metab 16, 478-488
12. Garruti G, Wang HH, Bonfrate L, de Bari O, Wang DQ and Portincasa P (2012) A pleiotropic role for the orphan nuclear receptor small heterodimer partner in lipid homeostasis and metabolic pathways. J Lipids 2012, 304292
13. Kerr TA, Saeki S, Schneider M et al (2002) Loss of nuclear receptor SHP impairs but does not eliminate negative feedback regulation of bile acid synthesis. Dev Cell 2, 713-720
14. Kim ND, Im EO, Choi YH and Yoo YH (2002) Synthetic bile acids: novel mediators of apoptosis. J Biochem Mol Biol 35, 134-141
15. Noshiro M, Usui E, Kawamoto T et al (2007) Multiple mechanisms regulate circadian expression of the gene for cholesterol 7alpha-hydroxylase (Cyp7a), a key enzyme in hepatic bile acid biosynthesis. J Biol Rhythms 22, 299-311
16. Park YK and Park H (2012) Differentiated embryo chondrocyte 1 (DEC1) represses PPARgamma2 gene through interacting with CCAAT/enhancer binding protein beta (C/EBPbeta). Mol Cells 33, 575-581
17. Choi SM, Cho HJ, Cho H, Kim KH, Kim JB and Park H (2008) Stra13/DEC1 and DEC2 inhibit sterol regulatory element binding protein-1c in a hypoxia-inducible factor-dependent mechanism. Nucleic Acids Res 36, 6372-6385
18. Ramakrishnan SK, Taylor M, Qu A et al (2014) Loss of von Hippel-Lindau protein (VHL) increases systemic cholesterol levels through targeting hypoxia-inducible factor 2alpha and regulation of bile acid homeostasis. Mol Cell Biol 34, 1208-1220
19. Shinkyo R and Guengerich FP (2011) Cytochrome P450 7A1 cholesterol 7alpha-hydroxylation: individual reaction steps in the catalytic cycle and rate-limiting ferric iron reduction. J Biol Chem 286, 4632-4643
20. Berchner-Pfannschmidt U, Tug S, Trinidad B et al (2008) Nuclear oxygen sensing: induction of endogenous prolyl-hydroxylase 2 activity by hypoxia and nitric oxide. J

Biol Chem 283, 31745-31753
21. Choi SM, Oh H and Park H (2008) Microarray analyses of hypoxia-regulated genes in an aryl hydrocarbon receptor nuclear translocator (Arnt)-dependent manner. FEBS J 275, 5618-5634
22. Lee HY, Choi K, Oh H, Park YK and Park H (2014) HIF-1-dependent induction of Jumonji domain-containing protein (JMJD) 3 under hypoxic conditions. Mol Cells 37, 43-50
23. Lee HY, Yang EG and Park H (2013) Hypoxia enhances the expression of prostate-specific antigen by modifying the quantity and catalytic activity of Jumonji C domain-containing histone demethylases. Carcinogenesis 34, 2706-2715
24. Hur E, Kim HH, Choi SM et al (2002) Reduction of hypoxia-induced transcription through the repression of hypoxia-inducible factor-1alpha/aryl hydrocarbon receptor nuclear translocator DNA binding by the 90-kDa heat-shock protein inhibitor radicicol. Mol Pharmacol 62, 975-982
25. Bhalla S, Ozalp C, Fang S, Xiang L and Kemper JK (2004) Ligand-activated pregnane X receptor interferes with HNF-4 signaling by targeting a common coactivator PGC-1alpha. Functional implications in hepatic cholesterol and glucose metabolism. J Biol Chem 279, 45139-45147
26. Lee J, Son J, Lee M, Lee KT and Kim DH (2003) Simultaneous quantitation of enalapril and enalaprilat in human plasma by 96-well solid-phase extraction and liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 17, 1157-1162