Original Paper

Hypoxia Induces Dysregulation of Lipid Metabolism in HepG2 Cells via Activation of HIF-2 α

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Key Words

HIF-2 α • HepG2 • Fatty acid metabolism • Cholesterol metabolism

Abstract

Background: Hypoxia is a risk factor for non-alcoholic fatty liver diseases, leading to permanent imbalance of liver lipid homeostasis and steatohepatitis. The current study examined the effect of HIF-2 α , an oxygen-sensitive heterodimeric transcription factor, on hypoxia-induced dysregulation of lipid metabolism in HepG2 cells. **Methods:** Studies were conducted in C57BL/6 male mice and human HepG2 cells under hypoxic conditions, transfected with HIF-2 α -targeted shRNA. The mRNA and protein expressions of key genes relevant to lipid metabolism were determined via RT-qPCR and western blot, respectively. Intracellular lipid accumulation was determined by Nile red, filipin staining and quantitative assay kits. **Results:** HIF-2 α protein was quantified in both HepG2 cells and C57BL/6 mice under hypoxic conditions. Intracellular lipid accumulation and increased lipid levels induced by hypoxia were significantly reduced by silence of HIF-2 α expression, associated with reversed expression of *ABCA1* and *ADRP*, key genes involved in cholesterol excretion and fatty acid uptake respectively. However, HIF-2 α had no effect on enzymatic activity and expression of key genes involved in fatty acid β -oxidation or cholesterol metabolism. **Conclusion:** Inhibition of HIF-2 α protein reversed lipid metabolism dysregulation induced by acute hypoxia in HepG2 cells, which suggested that HIF-2 α signaling may be relevant to oxygen-dependent lipid homeostasis in the liver.

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Introduction

Oxygen is crucial to the process of metabolism. A state of hypoxia, or lack of adequate oxygen, activates a variety of complex pathways at both the cellular and organ level to reinstate oxygen homeostasis. The liver is essential to metabolism, and is extremely sensitive to low-oxygen conditions. Lipid accumulation in the blood vessels and other tissues of the liver leads to an insufficient blood supply to hepatocytes and lipid peroxidation, setting up a hypoxic microenvironment; prolonged hypoxia can promote uncontrolled lipid accumulation [1, 2]. The liver's metabolic functions include lipid biosynthesis, uptake, catabolism, and reverse cholesterol transport. Lipid biosynthesis in the liver is regulated by sterol regulatory element-binding proteins (SREBPs), a family of transcription factors that include the isoforms SREBP-1a, SREBP-1c, and SREBP-2 [3]. SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of fatty acids, cholesterol, and triglycerides, whereas SREBP-1c preferentially regulates enzymes of fatty acid synthesis. Fatty acid synthase (FAS) is the principle target of SREBP-1c [4, 5]. SREBP-2 regulates cholesterol biosynthesis and uptake, in particular the low-density lipoprotein receptor (LDLR) [6, 7]. Another target of SREBP-2 is 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), the rate-limiting step and major control point of *de novo* cholesterol synthesis [8, 9].

Other genes important to lipid metabolism in the liver include adipose differentiation-related protein (ADRP), which participates in fatty acid uptake. Carnitine palmitoyltransferase-1 α (CPT-1 α), a target of peroxisome proliferator-activated receptor alpha (PPAR- α), controls mitochondrial fatty acid import [5]. Hydroxyacyl-CoA dehydrogenase (HADH) catalyzes the NAD⁺-dependent dehydrogenation step in fatty acid β -oxidation [10].

Cholesterol catabolism involves mainly the biodegradation of cholesterol to bile acids occurring in the hepatocyte endoplasmic reticulum, and begins with the transformation of cholesterol into 7- α -hydroxy cholesterol catalyzed by CYP7A1 (cytochrome P450 family 7 subfamily A polypeptide 1) [11-13]. Cholesterol clearance pathways are also mediated by members of the ATP-binding cassette (ABC) transporter family. An example is ABCA1 (ABC subfamily A member 1), which participates in transport of cholesterol in the hepatocyte to lipoprotein receptors and the formation of HDL cholesterol [14, 15]. ABCG5 and ABCG8 (ABC subfamily G members 5 and 8, respectively) regulate the excretion of liver cellular cholesterol into bile [16, 17].

The hypoxia-inducible factor (HIF) family of transcription factors has a central role in the main pathway associated with hypoxic stress [18, 19]. They participate in tissue adaptive responses to hypoxia by regulating the expression of hypoxia-induced genes, allowing survival within a large range of reduced oxygen concentrations. The HIF family are heterodimers composed of one α subunit that is unstable in the presence of relatively high levels of oxygen (above 5%), and one constitutively expressed β subunit (or ARNT [aryl hydrocarbon receptor nuclear translocator]). Mammals possess three HIF isoforms, HIF-1, HIF-2, and HIF-3.

Under normoxic conditions, HIF- α subunits are targeted for proteasomal degradation by the Von Hippel-Lindau (VHL) tumor suppressor (Pvhl) catalyzed by oxygen-dependent prolyl hydroxylase domain (PHD) proteins (PHD 1, 2 and 3). Under hypoxic conditions, HIF- α subunits are stabilized and translocate to the nucleus to activate transcription upon binding to hypoxia response elements located within regulatory elements of HIF-target genes.

HIF-2 α , also known as EPAS1 (*Homo sapiens* endothelial PAS domain protein 1), HLF (HIF-like factor), HRF (HIF-related factor), or MOP2 (member of PAS superfamily 2), was first discovered by Tian et al. in 1997 [20]. Recent studies have reported that many cellular processes such as angiogenesis, erythropoiesis, energy metabolism, cell migration, and tumor invasion are influenced by HIF-2 α . Since oxygen consumption in mitochondria seriously influences fatty acid oxidation during hypoxia, it is conceivable that lipid metabolism should be regulated through activation of HIFs. However, the effects of HIF-2 α and HIF-1 α on lipid metabolism have not been definitively determined.

In certain cell types, the expression of PPAR α and the DNA-binding affinity of PPAR α were reduced during hypoxia, in a HIF-1 α -dependent manner [21]. Moreover, HIF-1 α has been reported to induce the expression of HIG2, a lipid droplet protein involved in neutral lipid formation [22], and HIF-1 α was even considered a protective factor against alcoholic fatty liver [23]. In contrast, Rankin et al. [24] disclosed the importance of HIF-2 α rather than HIF-1 α in the regulation of lipid droplet formation and continuous lipid accumulation in VHL-deficient mouse liver. This conclusion was further supported by another study indicating that development of fatty liver disease in PHD2/3 knockout mice was under the control of HIF-2 α [25]. Considerable controversy remains regarding hepatic steatosis in HIF-2 α -deficient mice [26], and lipid accumulation stimulated by forced expression of HIF-1 α in mice [27].

Considering the controversies surrounding HIFs in lipid metabolism, it is important to understand all of the mechanisms of hypoxia-induced lipid dysregulation and clarify the roles of HIFs separately. In this study, we focused on whether HIF-2 α is associated with lipid metabolism under hypoxic conditions in cells of the human hepatoma cell line HepG2, and investigated the molecular processes activated within the cells.

Materials and Methods

C57BL/6 mouse liver ischemia-reperfusion (IR) model

The Ethics Committee of Nanjing Medical University approved this study. Six male C57BL/6 wild-type mice (age 10 weeks; 22–25 g) were purchased from Model Animal Research Center of Nanjing Medical University, Nanjing, China. The 6 mice were randomly apportioned to a control group or an ischemic-reperfusion (IR) group. Mice were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. The skin was disinfected with 1% iodine. The incision was mid-abdominal to allow for full liver exposure. For mice in the IR group, blood vessels were clamped with a non-invasive vascular clamp for one hour as described previously [28], which led to segmental (70%) hepatic ischemia, and then the liver was reperused for 6 h before tissues were harvested. Mice in the control group underwent the same procedure, but with no clamping of the blood vessels. The mice were euthanized by cutting the diaphragm and heart. Hepatic tissue was obtained and rapidly stored at -70°C for future analysis.

Cell culture

Cells of the human hepatocellular carcinoma cell line HepG2 were purchased from Shanghai Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Wisent, South America) with 15% fetal bovine serum (FBS; Wisent, South America). Cells were incubated in normoxic (21% O₂, 5% CO₂) or hypoxic (1% O₂, 5% CO₂, 94% N₂) conditions with a humidified chamber at 37 $^{\circ}\text{C}$.

HIF-2 α negative control and inhibitor, transient and stable transfection

To silence the expression of HIF-2 α , for stable transfection the plasmids pGPU6/GFP/Neo-HIF-2 α -homo-1250 and pGPU6/GFP/Neo-shNC were designed and synthesized by GenePharma (Shanghai, China; Table 1). For transient transfection, HIF-2 α -silencing small interfering RNA (siRNA; EPAS1-homo-2778) and negative control siRNA (siNC) were also obtained from GenePharma (Shanghai, China; Table 2).

For gene transfection, the cells were cultured in 6-well plates to 50% confluence. The plasmids or siRNAs were transfected into cells using Lipofectamine 2000 in accordance with the manufacturer's instructions (Invitrogen, USA). Cells were transfected for 6 h in medium without FBS. After changing to normal culture medium and culturing for 48 h, siRNA and siNC cells in transient transfection were prepared for further experiments. For stable transfection, 1 mg/mL G418 was incubated with cells for 14 d to obtain stable transfected cell lines (shRNA and shNC cells).

Real-time quantitative PCR

Total RNA from cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using the PrimeScript RT Kit (Takara) in accordance with the manufacturer's instructions. Concentrations were measured using a NanoDrop spectrophotometer (ND-1000V3.5.2 software, USA). RT-

Table 1. Sequences of HIF-2 α inhibitor and negative control for stable transfection

Primer sequences (5'→3')		
pGPU6/GFP/Neo-HIF-2 α -homo-1250	Sense	CACCGCCACAGCATGGACATG
		AAGTTTCAAGAGAACTTCATG
		TCCATGCTGTGGCTTTTGTG
	Antisense	GATCCAAAAAGCCACAGCAT
pGPU6/GFP/Neo-shNC	Sense	GGACATGAAGTTCTCTGAAA
		CTTCATGTCCATGCTGTGGC
		CACCGTTCTCCGAACGTGTAC
	Antisense	GTCAAGAGATTACGTGACACG
	Sense	TTCGGAGAATTTTGTG
		GATCCAAAAATTCTCCGAACG
		TGTCACGTAATCTCTGTACGTG
	Antisense	ACACGTTCCGGAGAAC

Table 2. Sequences of HIF-2 α inhibitor and negative control for transient transfection

Primer sequences (5'→3')		
EPAS1-homo-2778	Sense	GCGCAAAUGUACCCAAUGATT
	Antisense	UCAUUGGGUACAUAUUGCGCTT
siNC	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGAATT

Table 3. Sequences of target genes

Primer sequences (5'→3')		
HIF-2 α	F	ATGAAGAGCAAGCCTTCCAG
	R	TGGGGTTTTGGGTGAACCTTA
ADRP	F	CAGACCATTCTCAGCTCCA
	R	GGCAATTGCAAGAGTACGTG
CPT-1 α	F	ATGAGTCGTGCCACCAAGAT
	R	AAGAGGCCTCACCAGCTGTA
SREBP-2	F	CTACGGTGCAGACAGTTGCT
	R	CCACCAGGGTTGGTACTTGA
LDLR	F	TCTGCAACATGGCTAGAGACT
	R	TCCAAGCATTCGTTGGTCCC
HMGCR	F	TGATTGACCTTCCAGAGCAAG
	R	CTAAAATTGCCATTCCACGAGC
CYP7A1	F	GAGAAGGCAACGGGTGAAC
	R	GGATTGGCACCAGTTCGAGA
ABCA1	F	TAATGCCAACAACCCCTGTT
	R	GGAAACCAGAGAAGGTTTCA
ABCG5	F	GGTCTCCTTACCAGTTTGTTG
	R	ATATCCAAATCGGGCAACCT
ABCG8	F	AGAGACCTTGGCCTTCATTG
	R	GAAGCTGTCGAGCCCGA
GAPDH	F	CAGCCTCAAGATCATCAGCA
	R	TGTGGTCATGAGTCTTCCA
Hif-2 α (Mus)	F	GAGGAAGGAGAAATCCCGTGA
	R	TATGTGTCCGAAGGAAGCTGA
Gapdh (Mus)	F	CCTGGAGAAACCTGCCAAGTA
	R	TGGAAGAGTGGGAGTTGCTGT

qPCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche, USA) with an ABI StepOne machine (Applied Biosystems, Life Technologies, CA, USA). Briefly, qPCR cycling was performed as follows: initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 10 s; annealing for 60 s at 60 °C; and a melting curve profile set at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Transcript expression levels were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels as an endogenous control. Primers for qPCR were synthesized by Invitrogen (Shanghai, China; Table 3).

Western blot analysis

Proteins were prepared by homogenizing cells in RIPA buffer (Beyotime, China) containing a protease inhibitor cocktail mix from cOmplete ULTRA Tablets (Roche, Germany) on ice. The protein concentration was determined using a bicinchoninic acid protein assay kit (Keygen, Nanjing, China). Proteins were resolved via SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Temecula, CA, USA), blocked with milk for 2 h, probed with primary antibodies at 4 °C overnight, and incubated with secondary antibodies for 2 h at room temperature. The primary antibodies were: HIF-2 α (Abcam, Cambridge, MA, USA), HIF-1 α (human; Cell Signaling, USA), HIF-1 α (mus; Santa Cruz, USA), and SREBP-1 (Santa Cruz, USA), each used at a dilution of 1:200; FAS (Cell Signaling, USA) was diluted at 1:1000; β -actin and GAPDH from BioWorld (Irving, TX, USA) were diluted at 1:1000. Secondary antibodies were obtained from ZhongShan JinQiao (China). Protein levels were normalized to β -actin or GAPDH. Electrochemiluminescence was analyzed with a Chemilmager 5500 imaging system (Alpha Innotech, San Leandro, CA, USA).

Analysis of lipid accumulation via Nile red staining

HepG2 cells were plated in 6-well plates. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min and then rinsed twice with PBS. The cells were stained with Nile red (1 μ g/mL) in 60% 2-propanol for 5 min and washed three times with PBS. Images were taken with an inverted fluorescence microscope (Olympus, Tokyo, Japan) equipped with an image recorder under a 10 $\times$ lens.

Analysis of free cholesterol accumulation via Filipin staining

HepG2 cells at a concentration of 1×10^4 /mL were cultured on 22 mm $\times$ 22 mm coverslips in 6-well plates overnight. According to group, the corresponding treatment was given. Cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min at 4 °C. They were subsequently treated with 0.1%

Triton for 3 min, and washed three times with PBS. Cells were stained with 50 μ g/mL Filipin III fluorescent dye at 37 °C for 30 min and washed with PBS three times. Images were obtained with a fluorescent microscope (Olympus) at 10 $\times$ lens.

Measurement of intracellular free cholesterol and triglyceride levels

HepG2 cells were collected and homogenized in RIPA buffer. Intracellular triglyceride and free cholesterol were measured by using triglyceride and free cholesterol assay kits (Biovision, USA), in accordance with the kit manuals. Triglyceride and cholesterol levels were normalized to total protein concentrations.

Measurements of HADH activity

HADH activity was measured as described previously [29, 30]. Briefly, HepG2 cell pellets were homogenized in 1:10 wt/vol homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 10% glycerol [wt/vol], 0.1% Triton-100). After homogenization, the cell lysates were centrifuged at 8000 rpm for 30 min at 4 °C. In each well of a 96-well UV plate, 260 μ L imidazole (50 mM, pH 7.4), 20 μ L reduced nicotinamide adenine dinucleotide (0.15 mM, $\epsilon = 6.22 \text{ mol}\cdot\text{mL}^{-1}\cdot\text{cm}^{-1}$), and 10 μ L cell lysate were added. The absorbance was detected at 30 °C with a SpectraMax 190 spectrophotometer (Molecular Devices), at 340 nm for 5 min with 30-s intervals both before (baseline) and after adding 10 μ L of 3 mM acetoacetyl CoA. HADH activity was normalized to total protein concentrations.

Measurements of Cholesterol Efflux

Cholesterol efflux was measured as described previously [31]. Briefly, HepG2 cells were cultured in DMEM media containing 15% FBS, trace labeled with 1 μ Ci/ml ^3H -cholesterol (Perkin-Elmer, USA) overnight. Before treatment, cells were washed with PBS and equilibrated in media for 2 h. Cells were then incubated under normoxia or hypoxia conditions for 24 h, in serum free DMEM containing 10 μ g/ml apoA-I and 2 mg/ml fatty acid-free BSA for 24 h. Supernatants were collected, centrifuged to remove cell debris, and radioactivity was quantified by scintillation counting (Microbeta2, Perkin Elmer). Cells were washed with PBS and lysed with 0.1 M NaOH, and cell lysates were analyzed by scintillation counting. Cholesterol efflux was calculated by cpm in media/(cpm in media+cpm in cellular lipid) and expressed in percent.

Statistical analysis

The equation $2^{-\Delta\Delta\text{CT}}$ was applied to calculate the fold changes of gene expression. Data are presented as the mean $\pm$ standard deviation for at least three separate experiments. Statistical analysis was performed using the paired 2-tailed Student's *t* test. A *P*-value of <0.05 was considered statistically significant.

Results

HIF-2 α expression in HepG2 cells in vitro and in C57BL/6 mice in vivo

To assess the role of HIF-2 α in lipid metabolism, we first determined the HIF-2 α mRNA and protein levels in HepG2 cells under normoxia and hypoxia (Fig. 1A, 1C). HIF-2 α protein expression increased in hypoxia over time, *in vitro* reaching a maximum at 24 h of hypoxia. However, there was no change in HIF-2 α mRNA levels.

To confirm our *in vitro* data, we created mouse liver models of ischemic-reperfusion. RNAs and protein was extracted from hepatic tissues as described in Methods. RT-qPCR and western blot analysis both revealed a slight but statistically significant increase in HIF-2 α expression in IR mice compared to controls (Fig. 1B, 1D). We also determined quantities of HIF-1 α protein both *in vitro* and *in vivo* and obtained similar results (Fig. 1E, 1F).

Establishment of HIF-2 α -silenced HepG2 cell lines

In the present study, we established stable HIF-2 α -silenced (shRNA) and negative control (shNC) cell lines by selecting transfected cells with G418. The transfection efficiency was 35% to 40%. The knockdown efficiency was confirmed by western blot analysis (Fig. 2A).

Fig. 1. HIF-2 α and HIF-1 α expression in HepG2 cells *in vitro* and in C57BL/6 mice *in vivo*. (A, C, E) HepG2 cells were cultured under hypoxic (1% O₂) conditions for 6, 12 and 24 h (group H). Total cell lysates were obtained to determine intracellular HIF-2 α and HIF-1 α expressions by RT-qPCR and western blot analysis (n = 3). (B, D, F) C57BL/6 mice were treated with ischemia-reperfusion for 6 h (group IR). Liver HIF-2 α and HIF-1 α expressions were detected via RT-qPCR and western blot analysis (n = 3). Each immunoblot is representative of three separate experiments. GAPDH and β -actin were used as internal controls. **P* < 0.05, IR group compared with untreated control groups.

Fig. 2. Establishment of HIF-2 α -silenced HepG2 cell lines. Cells were cultured in 6-well plates and transfected with pGPU6 plasmid DNA (4 μ g) or HIF-2 α siRNA (100 pM) by Lipofectamine 2000. After 6 h incubation, the culture media were changed to normal media and kept for 48 h until treated. For the stable transfection, cells were treated with 1 mg/mL G418 for 14 d. Cells were cultured under normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 h, and intracellular HIF-2 α and HIF-1 α protein expressions were detected by western blot analysis in total cell lysates. (A, C) HIF expressions of stable transfection (B, D) HIF expressions of transient transfection. Each immunoblot is representative of three separate experiments. GAPDH was used as an internal control.

Fig. 3. Effect of hypoxia and HIF-2 α on lipid accumulation. HepG2 cells were plated in 6-well plates and transfected with HIF-2 α siRNA (100 pM) by Lipofectamine 2000 for 6 h. After 48 h incubation, cells were cultured under normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 h, then fixed with 3.7% formaldehyde for 30 min. Cells were stained by Nile red (1 $\mu\text{g}/\text{mL}$) for 5 min or Filipin III (50 $\mu\text{g}/\text{mL}$) for 30 min as described in Materials and Methods, with images obtained by fluorescence microscopy. (A) Nile red staining for lipid accumulation, detected by FITC filter (10 $\times$). (B) Filipin staining for free cholesterol accumulation, detected by DAPI filter (10 $\times$). Each image is representative of three separate experiments. The scale bar is 100 μm . (C, D) The shNC and HIF-2 α -silenced HepG2 cells were cultured under normoxic or hypoxic conditions for 24 h. The intracellular triglyceride and free cholesterol levels were determined using Biovision kits as described under Materials and Methods. * $P < 0.05$; ** $P < 0.01$, shNC cell hypoxia group compared with normoxia group. # $P < 0.05$, shRNA cells compared with shNC cells under hypoxia.

Similarly, the efficiency of knockdown in transient transfection was also confirmed by western blot (Fig. 2B). Western blot analyses showed that cells in which RNA interference (RNAi) had been used had lower levels of HIF-2 α protein under hypoxic conditions. However, levels in shNC and siNC cells were comparable to the corresponding controls. As expected, minor efficiency of RNA interference was demonstrated under normoxic conditions.

We detected HIF-1 α expression in HepG2 cells to verify the specificity of designed siRNA and shRNA, which was not reversed by HIF-2 α shRNA or siRNA (Fig. 2C, 2D). These results indicated that the siRNA/shRNA used in our study specifically targeted HIF-2 α mRNA, without an effect on the expression of HIF-1 α .

Lipid accumulation measured by staining and quantification

To investigate lipid accumulation in HepG2 cells, we used Nile red staining. Nile red fluorescent dye binds to a wide variety of lipids including triacylglycerol and most fatty acids [32]. Under

Fig. 4. Effect of hypoxia on key genes involved in fatty acid metabolism and HADH enzymatic activity. The shNC HepG2 cells were cultured under normoxic (21% O₂) and hypoxic (1% O₂) conditions for 24 h, with total cell lysates and RNA obtained. Expressions of key genes relevant to fatty acid metabolism were determined by RT-qPCR and western blot (n=3). (A) *ADRP* mRNA; (B) *CPT-1 α* mRNA; (C) FAS and SREBP-1 α protein, each immunoblot is representative of three separate experiments. GAPDH was used as an internal control. (D) HADH enzymatic activities examined as described in Materials and Methods (n = 3). **P* < 0.05, hypoxia group compared with normoxia group.

normoxic conditions, the fluorescence levels of the three groups were not different (Fig. 3A). Twenty-four hours in a hypoxic environment induced marked lipid accumulation in HepG2 cells relative to the control cells that were cultured in normoxic conditions. Comparing siRNA and control cells under hypoxic environments, the results suggested that the accumulation effect was partly inhibited by silencing of HIF-2 α , judging from the decreased fluorescence intensity. No significant differences were observed between siNC and the control cells.

We also detected free cholesterol accumulation by means of filipin staining (Fig. 3B). The results were similar to those obtained by Nile red staining. Compared to the control group, blue fluorescence obviously increased under 24-h of hypoxia, evidence of increased cholesterol accumulation. However, under hypoxic conditions the amount of fluorescence increased less in siRNA cells than in the control group, and the degree of fluorescence of siNC cells and that of the control group were very similar. These results suggest that cholesterol accumulation induced by hypoxia can be partly reversed by inhibition of HIF-2 α .

Similar results were obtained in the quantitative measurements of lipid levels in HepG2 cells (Fig. 3C, 3D). The increased triglyceride and free cholesterol levels under hypoxic conditions were significantly prevented by silence of HIF-2 α expression, compared with the shNC control group.

Hypoxia regulated fatty acid metabolism in HepG2 cells

Expression of *ADRP* mRNA increased 3.3-fold after 24 h of hypoxia, while *CPT-1 α* mRNA expression was reduced by 45.1% (*P* < 0.05, both; Fig. 4A, 4B). SREBP-1 α and FAS protein levels appeared to be unaffected by hypoxic conditions (Fig. 4C). HADH enzymatic activity, a measure of β -oxidation, was found to be remarkably inhibited by hypoxic conditions (Fig. 4D).

Fig. 5. Effect of hypoxia on key genes involved in cholesterol metabolism. The shNC HepG2 cells were cultured under normoxic (21% O₂) and hypoxic (1% O₂) conditions for 24 h. The mRNA expressions of key genes relevant to cholesterol metabolism were determined by RT-qPCR analysis (n = 3). (A) *ABCA1*; (B) *ABCG5*; (C) *ABCG8*; (D) *CYP7A1*; (E) *SREBP-2*; (F) *LDLR*; (G) *HMGCR*. *GAPDH* was used as an internal control. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, hypoxia group compared with normoxia group.

Hypoxia regulated cholesterol metabolism in HepG2 cells

In the case of cholesterol accumulation, genes involved in catabolism (*ABCA1*, *ABCG5*, *ABCG8*, *CYP7A1*) all low expressed under hypoxia for 24 h, which dropped to 13.2%, 24.0%, 23.4%, 4.8%, respectively (Fig. 5A-D). Unexpectedly, the mRNA expression of genes involved in anabolism (*SREBP-2*, *LDLR*, and *HMGCR*) also were reduced 65.7%, 49.6% and 89.0% under hypoxic conditions (Fig. 5E, 5F, 5G).

HIF-2 α regulated fatty acid metabolism in HepG2 cells under hypoxia

To explore the cellular mechanism involved in fatty acid regulation by HIF-2 α , we examined via RT-qPCR in shRNA cells the key genes responsible for lipid uptake and

Fig. 6. Effect of HIF-2 α on key genes involved in fatty acid metabolism and HADH enzymatic activity. The shNC and HIF-2 α -silenced HepG2 cells were cultured under normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 h. The mRNA expressions of key genes relevant to fatty acid metabolism were determined by RT-qPCR (n = 3). (A) *ADRP*; (B) *CPT-1 α* . *GAPDH* was used as an internal control. (C) HADH enzymatic activities examined as described in Materials and Methods (n = 3). **P* < 0.05 shNC cells hypoxia group compared with normoxia group. # *P* < 0.05, shRNA cells compared with shNC cells under hypoxia.

β -oxidation. Compared to the shNC group, *ADRP* mRNA expression was reduced by 33% in the shRNA group under hypoxic conditions (Fig. 6A). There was no difference in *CPT-1 α* mRNA levels between the two groups under hypoxic conditions, nor in HADH enzymatic activity (Fig. 6B, 6C).

HIF-2 α regulated cholesterol excretion in HepG2 cells under hypoxic conditions

To further investigate the possible pathway controlled by HIF-2 α in cholesterol metabolism, we also evaluated by RT-qPCR the expression of key genes involved in cholesterol uptake, *de novo* synthesis, degradation, and reverse cholesterol transportation. Our data indicated that *ABCA1* mRNA had a slight but significant increase when HIF-2 α was silenced under hypoxia (*P* < 0.05; Fig. 7A). The cholesterol efflux relevant to hypoxia was also reversed obviously (Fig. 7H). However, between the shRNA and corresponding shNC group there were no significant differences in the mRNA expression of the other genes of interest (*SREBP-2*, *HMGCR*, *LDLR*, *CYP7A1*, all *ABCG5/8*; Fig. 7B-G).

Discussion

Dysregulation of lipid metabolism is increasingly recognized as a risk factor for many metabolic diseases, including nonalcoholic fatty liver disease and cardiovascular disease. Currently it is undecided whether obstructive sleep apnea syndrome affects serum lipids [33-37], but studies suggest that hypoxia may influence lipid metabolism. HIFs are important

Fig. 7. Effect of HIF-2 α on cholesterol metabolism and efflux. The shNC and HIF-2 α -silenced HepG2 cells were cultured in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 h. The mRNA expression of key genes relevant to cholesterol metabolism were determined by RT-qPCR analysis (n = 3). (A) *ABCA1*; (B) *ABCG5*; (C) *ABCG8*; (D) *CYP7A1*; (E) *SREBP-2*; (F) *LDLR*; (G) *HMGCR*. *GAPDH* was used as an internal control. (H) Cell cholesterol efflux were determined as described in *Materials and Methods* (n = 3). Percentage was calculated by cpm in media/(cpm in media+cpm in cellular lipid). **P* < 0.05; ***P* < 0.01; ****P* < 0.001, shNC cell hypoxia group compared with normoxia group. # *P* < 0.05, shRNA cells compared with shNC cells under hypoxia.

transcription factors with crucial roles in allowing adaption to hypoxic environments in mammals. In the present study, we investigated the effect of HIF-2 α on lipid metabolism and the potential underlying molecular mechanisms.

We detected the expressions of HIF-2 α in HepG2 cells *in vitro* and in C57BL/6 male mice. Under hypoxic conditions, HIF-2 α mRNA and protein levels were remarkably higher both *in vitro* and *in vivo*, except that mRNA expressed *in vitro* was not affected by 24-h hypoxic conditions. Data obtained from our animal experiments would be better supported by a larger sample than provided at this time. Two studies conducted by Wiesener et al. [38, 39] that support our findings also showed that hypoxia has an effect on HIF-2 α at the post-transcriptional level rather than the transcriptional level.

In the current study, we observed that lipid accumulation, including fatty acid and cholesterol, obviously increased under hypoxic conditions. Furthermore, we used siRNAs to specifically inhibit HIF-2 α production in the human hepatic carcinoma cell line, HepG2. Results from quantitative analysis and staining showed that both triglyceride and free cholesterol accumulation were augmented by HIF-2 α knockdown. Therefore, one possible explanation of hypoxia-induced lipid accumulation in hepatocytes is via a HIF-2 α transcription factor pathway, consistent with previous studies using mouse models [26, 40].

We also investigated the role of transcription factor induction of specific target genes with a variety of functions related to lipid metabolism and the hypoxic response. Our data suggest that the development of steatosis in HepG2 cells is a consequence of increased lipid uptake and reduced lipid β -oxidation. Hu et al. [41] already demonstrated that ADRP coordinates regulation of HIF-1 α and HIF-2 α in clear cell renal cancer cells. Also, ADRP was found necessary for lipid storage in fibroblasts and a variety of fatty liver disease models [14-16].

Unexpectedly, SREBP-1a and FAS, two key fatty acid synthesis relevant proteins induced by hypoxic conditions [42, 43] were not changed in our model. With further analysis, we found that HIF-2 α significantly upregulated the uptake of fatty acid (ADRP) expression. Finally, we found that reduced β -oxidation was not regulated by HIF-2 α , although inhibition of HIF-2 α under hypoxia resulted in a slight increase in *CPT-1 α* mRNA expression. As a reflection of β -oxidation efficiency, HADH enzyme activities were significantly decreased under hypoxic conditions, which also were not affected by HIF-2 α . Taken together, these data suggest that during hypoxia, ADRP signaling may be regulated by HIF-2 α to participate in fatty acid accumulation. This is similar to the conclusions of Rankin et al. [24].

With regard to cholesterol metabolism, in the present study hypoxia was associated with an obvious decline in expression levels of the key genes in HepG2 cells. The data suggest that hypoxia leads to accumulation of cholesterol via a decrease in biodegradation and reverse transportation. Interestingly, we found that expression of *SREBP-2* varied with oxygen concentration, but presented a downward trend under hypoxia. *LDLR* and *HMGCR*, which are downstream of *SREBP-2*, also showed similar trends. Our data is in disaccord with that of Li et al. [44, 45] who concluded that SREBP-2 signaling, as well as the SREBP-2 target *LDLR*, are not relevant to oxygen concentration in obese mice. With further analysis, we found that only *ABCA1* mRNA was significantly elevated by the inhibition of HIF-2 α during hypoxic conditions. Furthermore, we validated that the hypoxia related cholesterol excretion was also regulated by HIF-2 α . Other pathways affected by hypoxia may be through other mechanisms. One potential pathway mediated by HIF-1 α was suggested by Parathath et al. [46] and Manalo et al. [47]. Endoplasmic reticulum stress may also participate in regulation of lipid metabolism [48-51].

In conclusion, inhibition of HIF-2 α protein reversed hypoxia-induced lipid accumulation in HepG2 cells by decreasing the expression of ADRP, involved in fatty acid uptake, increasing *ABCA1* expression and relevant cholesterol excretion. These results suggest a possible mechanism for lipid metabolism dysregulation induced by acute hypoxia. These findings indicate that HIF-2 α signaling is relevant to oxygen-dependent lipid homeostasis in the liver, and may have implications for the therapeutic treatment of fatty liver and cardiovascular diseases.

Disclosure Statement

All authors report no conflicts of interest.

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References

- 1 Byrne CD: Hypoxia and non-alcoholic fatty liver disease. *Clin Sci (Lond)* 2010;118:397-400.
- 2 Semenza GL: Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol* 2014;9:47-71.
- 3 Higuchi N, Kato M, Shundo Y, Tajiri H, Tanaka M, Yamashita N, Kohjima M, Kotoh K, Nakamuta M, Takayanagi R, Enjoji M: Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. *Hepatol Res* 2008;38:1122-1129.
- 4 Magana MM, Koo SH, Towle HC, Osborne TF: Different sterol regulatory element-binding protein-1 isoforms utilize distinct co-regulatory factors to activate the promoter for fatty acid synthase. *J Biol Chem* 2000;275:4726-4733.
- 5 Nguyen P, Leray V, Diez M, Serisier S, Le Bloc'h J, Siliart B, Dumon H: Liver lipid metabolism. *J Anim Physiol Anim Nutr (Berl)* 2008;92:272-283.
- 6 Bengoechea-Alonso MT, Ericsson J: SREBP in signal transduction: cholesterol metabolism and beyond. *Curr Opin Cell Biol* 2007;19:215-222.
- 7 Horton JD, Goldstein JL, Brown MS: SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125-1131.
- 8 Burg JS, Espenshade PJ: Regulation of HMG-CoA reductase in mammals and yeast. *Prog Lipid Res* 2011;50:403-410.
- 9 Jo Y, Debose-Boyd RA: Control of cholesterol synthesis through regulated ER-associated degradation of HMG CoA reductase. *Crit Rev Biochem Mol Biol* 2010;45:185-198.
- 10 Yang SY, He XY, Schulz H: 3-Hydroxyacyl-CoA dehydrogenase and short chain 3-hydroxyacyl-CoA dehydrogenase in human health and disease. *FEBS J* 2005;272:4874-4883.
- 11 Gilardi F, Mitro N, Godio C, Scotti E, Caruso D, Crestani M, De Fabiani E: The pharmacological exploitation of cholesterol 7 α -hydroxylase, the key enzyme in bile acid synthesis: from binding resins to chromatin remodelling to reduce plasma cholesterol. *Pharmacol Ther* 2007;116:449-472.
- 12 Gupta S, Stravitz RT, Dent P, Hylemon PB: Down-regulation of cholesterol 7 α -hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway. *J Biol Chem* 2001;276:15816-15822.
- 13 Noshiro M, Usui E, Kawamoto T, Kubo H, Fujimoto K, Furukawa M, Honma S, Makishima M, Honma K, Kato Y: Multiple mechanisms regulate circadian expression of the gene for cholesterol 7 α -hydroxylase (Cyp7a), a key enzyme in hepatic bile acid biosynthesis. *J Biol Rhythms* 2007;22:299-311.
- 14 Field FJ, Watt K, Mathur SN: Origins of intestinal ABCA1-mediated HDL-cholesterol. *J Lipid Res* 2008;49:2605-2619.
- 15 Wang N, Tall AR: Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* 2003;23:1178-1184.
- 16 Dijkers A, Freak de Boer J, Annema W, Groen AK, Tietge UJ: Scavenger receptor BI and ABCG5/G8 differentially impact biliary sterol secretion and reverse cholesterol transport in mice. *Hepatology* 2013;58:293-303.

- 17 Kidambi S, Patel SB: Cholesterol and non-cholesterol sterol transporters: ABCG5, ABCG8 and NPC1L1: a review. *Xenobiotica* 2008;38:1119-1139.
- 18 Majmundar AJ, Wong WJ, Simon MC: Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* 2010;40:294-309.
- 19 Patel SA, Simon MC: Biology of hypoxia-inducible factor-2 α in development and disease. *Cell Death Differ* 2008;15:628-634.
- 20 Tian H, McKnight SL, Russell DW: Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* 1997;11:72-82.
- 21 Narravula S, Colgan SP: Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor α expression during hypoxia. *J Immunol* 2001;166:7543-7548.
- 22 Gimm T, Wiese M, Teschemacher B, Deggerich A, Schodel J, Knaup KX, Hackenbeck T, Hellerbrand C, Amann K, Wiesener MS, Honing S, Eckardt KU, Warnecke C: Hypoxia-inducible protein 2 is a novel lipid droplet protein and a specific target gene of hypoxia-inducible factor-1. *FASEB J* 2010;24:4443-4458.
- 23 Nishiyama Y, Goda N, Kanai M, Niwa D, Osanai K, Yamamoto Y, Senoo-Matsuda N, Johnson RS, Miura S, Kabe Y, Suematsu M: HIF-1 α induction suppresses excessive lipid accumulation in alcoholic fatty liver in mice. *J Hepatol* 2012;56:441-447.
- 24 Rankin EB, Rha J, Selak MA, Unger TL, Keith B, Liu Q, Haase VH: Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. *Mol Cell Biol* 2009;29:4527-4538.
- 25 Minamishima YA, Moslehi J, Padera RF, Bronson RT, Liao R, Kaelin WG Jr: A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. *Mol Cell Biol* 2009;29:5729-5741.
- 26 Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA: Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *Epas1*^{-/-} mice. *Nat Genet* 2003;35:331-340.
- 27 Kim WY, Safran M, Buckley MR, Ebert BL, Glickman J, Bosenberg M, Regan M, Kaelin WG Jr: Failure to prolyl hydroxylate hypoxia-inducible factor α phenocopies VHL inactivation in vivo. *EMBO J* 2006;25:4650-4662.
- 28 Abe Y, Hines IN, Zibari G, Pavlick K, Gray L, Kitagawa Y, Grisham MB: Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo. *Free Radic Biol Med* 2009;46:1-7.
- 29 Ito M, Jaswal JS, Lam VH, Oka T, Zhang L, Beker DL, Lopaschuk GD, Rebeyka IM: High levels of fatty acids increase contractile function of neonatal rabbit hearts during reperfusion following ischemia. *Am J Physiol Heart Circ Physiol* 2010;298:H1426-1437.
- 30 Shrivastav S, Zhang L, Okamoto K, Lee H, Lagranha C, Abe Y, Balasubramanyam A, Lopaschuk GD, Kino T, Kopp JB: HIV-1 Vpr enhances PPAR β /delta-mediated transcription, increases PDK4 expression, and reduces PDC activity. *Mol Endocrinol* 2013;27:1564-1576.
- 31 Rohrl C, Eigner K, Winter K, Korbelius M, Obrowsky S, Kratky D, Kovacs WJ, Stangl H: Endoplasmic reticulum stress impairs cholesterol efflux and synthesis in hepatic cells. *J Lipid Res* 2014;55:94-103.
- 32 Greenspan P, Mayer EP, Fowler SD: Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 1985;100:965-973.
- 33 Coughlin SR, Mawdsley L, Mugarza JA, Calverley PM, Wilding JP: Obstructive sleep apnoea is independently associated with an increased prevalence of metabolic syndrome. *Eur Heart J* 2004;25:735-741.
- 34 Drager LF, Bortolotto LA, Maki-Nunes C, Trombetta IC, Alves MJ, Fraga RF, Negrao CE, Krieger EM, Lorenzi-Filho G: The incremental role of obstructive sleep apnoea on markers of atherosclerosis in patients with metabolic syndrome. *Atherosclerosis* 2010;208:490-495.
- 35 Roche F, Sforza E, Pichot V, Maudoux D, Garcin A, Celle S, Picard-Kossovsky M, Gaspoz JM, Barthelemy JC, Group PS: Obstructive sleep apnoea/hypopnea influences high-density lipoprotein cholesterol in the elderly. *Sleep Med* 2009;10:882-886.
- 36 Tan KC, Chow WS, Lam JC, Lam B, Wong WK, Tam S, Ip MS: HDL dysfunction in obstructive sleep apnea. *Atherosclerosis* 2006;184:377-382.
- 37 Tokuda F, Sando Y, Matsui H, Koike H, Yokoyama T: Serum levels of adipocytokines, adiponectin and leptin, in patients with obstructive sleep apnea syndrome. *Intern Med* 2008;47:1843-1849.
- 38 Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU: Widespread hypoxia-inducible expression of HIF-2 α in distinct cell populations of different organs. *FASEB J* 2003;17:271-273.

- 39 Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH: Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1 α . *Blood* 1998;92:2260-2268.
- 40 Wada T, Shimba S, Tezuka M: Transcriptional regulation of the hypoxia inducible factor-2 α (HIF-2 α) gene during adipose differentiation in 3T3-L1 cells. *Biol Pharm Bull* 2006;29:49-54.
- 41 Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC: Differential roles of hypoxia-inducible factor 1 α (HIF-1 α) and HIF-2 α in hypoxic gene regulation. *Mol Cell Biol* 2003;23:9361-9374.
- 42 Guillet-Deniau I, Pichard AL, Kone A, Esnous C, Nieruchalski M, Girard J, Prip-Buus C: Glucose induces de novo lipogenesis in rat muscle satellite cells through a sterol-regulatory-element-binding-protein-1c-dependent pathway. *J Cell Sci* 2004;117:1937-1944.
- 43 Shimomura I, Bashmakov Y, Horton JD: Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem* 1999;274:30028-30032.
- 44 Li J, Grigoryev DN, Ye SQ, Thorne L, Schwartz AR, Smith PL, O'Donnell CP, Polotsky VY: Chronic intermittent hypoxia upregulates genes of lipid biosynthesis in obese mice. *J Appl Physiol* (1985) 2005;99:1643-1648.
- 45 Li J, Thorne LN, Punjabi NM, Sun CK, Schwartz AR, Smith PL, Marino RL, Rodriguez A, Hubbard WC, O'Donnell CP, Polotsky VY: Intermittent hypoxia induces hyperlipidemia in lean mice. *Circ Res* 2005;97:698-706.
- 46 Parathath S, Mick SL, Feig JE, Joaquin V, Grauer L, Habel DM, Gassmann M, Gardner LB, Fisher EA: Hypoxia is present in murine atherosclerotic plaques and has multiple adverse effects on macrophage lipid metabolism. *Circ Res* 2011;109:1141-1152.
- 47 Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, Semenza GL: Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* 2005;105:659-669.
- 48 Cao R, Hu Y, Wang Y, Gurley EC, Studer EJ, Wang X, Hylemon PB, Pandak WM, Sanyal AJ, Zhang L, Zhou H: Prevention of HIV protease inhibitor-induced dysregulation of hepatic lipid metabolism by raltegravir via endoplasmic reticulum stress signaling pathways. *J Pharmacol Exp Ther* 2010;334:530-539.
- 49 Colgan SM, Hashimi AA, Austin RC: Endoplasmic reticulum stress and lipid dysregulation. *Expert Rev Mol Med* 2011;13:e4.
- 50 Lee AH, Glimcher LH: Intersection of the unfolded protein response and hepatic lipid metabolism. *Cell Mol Life Sci* 2009;66:2835-2850.
- 51 Zheng Z, Zhang C, Zhang K: Role of unfolded protein response in lipogenesis. *World J Hepatol* 2010;2:203-207.