INHIBITION OF CHOLESTEROL BIOSYNTHESIS UNDER HYPOXIA

A Thesis

by

QIULIN TAN

Submitted to the Office of Graduate Studies of Texas A&M University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Toxicology
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ABSTRACT

Inhibition of Cholesterol Biosynthesis under Hypoxia. (December 2005)

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Oxygen balance is very important and tightly regulated in mammals. Under hypoxia, hypoxia inducible factor 1β (HIF-1β) dimerizes with hypoxia inducible factor 1α (HIF-1α) and activates expression of several genes. Using a mammalian two hybrid assay, we found that HIF-1β interacted with sterol response element binding protein 1a (SREBP1a). SREBP1a regulates transcription of HMG-CoA reductase via binding to the sterol response element (SRE) in the promoter region. HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis. The interaction between SREBP1a and HIF-1β suggests that HIF-1β may play an important role in regulation of cholesterol biosynthesis. We tested the effects of hypoxia on the HMG-CoA reductase. We found that hypoxia caused suppression of SRE-driven luciferase reporter gene expression. HMG-CoA reductase mRNA levels decreased under hypoxia in both hepatoma cells and mouse primary hepatocytes. Electrophoretic mobility shift assay showed that HIF-1β blocked binding of SREBP1a to the SRE sequence in vitro. Ectopic expression of HIF-1β suppressed the SRE-driven luciferase reporter gene expression in BPR cells (HIF-1β-/-). Our results suggest that hypoxia inhibits cholesterol biosynthesis by suppressing SREBP1a-regulated
gene expression and this suppression is caused by the blockage of SREBP1a binding to SRE sequence by HIF-1β.
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CHAPTER I
INTRODUCTION

1.1 Hypoxia signaling pathway

Oxygen balance in mammals is tightly regulated so as to maintain sufficient levels for oxygen-dependent processes, while minimizing the production of reactive oxygen species (ROS) that can cause oxidative damage to DNA, lipids and protein. Under hypoxia, where oxygen supplies are insufficient, a physiological response is induced which increases the capacity of blood to carry oxygen to tissues, and alters cellular metabolism. For example, anaerobic glycolysis is induced to produce more ATP to meet the energy demand (Wenger, 2000). The hypoxia-inducible factors (HIFs) are key transcriptional regulators in response to this hypoxic state in both adult and embryonic organisms. Under hypoxia, mammals increase the expression of a large variety of genes, including erythropoietin, vascular endothelial growth factor (VEGF), and glycolytic enzymes, to stimulate erythropoiesis, angiogenesis, and glycolysis (Bracken et al., 2003).

This thesis follows the style of Gene.
HIF-1α (Hypoxia-induced factor-1α) protein stability can be regulated by oxygen levels (Jaakkola et al., 2001). Under normoxia, HIF-prolyl-4-hydroxylases (HPH), also designated as prolyl hydroxylase domain (PHD), hydroxylate specific proline residues of HIF-1α in an oxygen and iron-dependent manner. Hydroxylated HIF-α protein binds to the von-Hippel-Lindau (VHL) protein which is a component of an E3 ubiquitin ligase complex. HIF-α is subsequently ubiquitylated, and degraded by the proteasome (Wenger, 2002). In hypoxia, PHD/HPH activity is blocked because HIF-1α proline cannot be hydroxylated and bind to VHL protein due to oxygen insufficiency, and HIF-1α protein degradation is repressed.

The transcriptional activity of HIF-1α is also regulated by oxygen level (Jaakkola et al., 2001). Under normoxia, HIF-1α asparaginyl hydroxylase (FIH-1) binds and hydroxylates specific asparagine residues of HIF-1α in an oxygen and iron-dependent way. This blocks the recruitment of transcriptional coactivators (p300/CBP) by the carboxy-terminal transactivation domain (C-TAD), therefore resulting in transcriptional inactivation of HIF-1α (Lando et al., 2002). Under hypoxia, FIH-1 activity is blocked due to oxygen insufficiency, leading to asparagine hydroxylation deficiency, and thus enhancement of coactivator recruitment and target gene expression.

Under hypoxia, HIF-1α becomes stabilized, translocates to the nucleus, and dimerizes with HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) to induce gene expression (Gu et al., 2000). HIF-1α and
HIF-1β belong to a family of transcriptional factor basic helix-loop-helix (bHLH)/PAS proteins. HIF-1α / HIF-1β heterodimers bind to Hypoxia response elements (HREs) (A/G) CGTG in the regulatory regions of various target genes to activate gene expression (Figure 1). These target genes include erythropoietin (Semenza and Wang, 1992), vascular endothelial growth factor (Forsythe et al., 1996), insulin-like growth factor 2 (Feldser et al., 1999), glucose transporter-1 (Ebert et al., 1995) and Differentially expressed in chondrocytes protein 1 (DEC1/) (Miyazaki et al., 2002), etc.

1.2 Cholesterol biosynthesis and sterol response element binding proteins (SREBPs)

Cholesterol homeostasis is very important for human or animals. Cholesterol must be supplied for many physiological functions such as cell membrane construction and biosynthesis of vital hormones. However, excess cholesterol must be avoided because it can form solid crystals which can be toxic to cells. Excess cholesterol can also cause many adverse effects such as atherosclerosis or neurological degenerative disease. Therefore, cholesterol biosynthesis must be tightly regulated and this is mainly achieved through an end-product feedback repression of genes including HMG-CoA reductase, HMG CoA synthase and low density lipoprotein (LDL) receptor (Reinhart et al., 1987). These genes govern the synthesis of cholesterol or its receptor-mediated uptake.
Fig. 1. Hypoxia signaling pathway. (Gu, et al., 2000). Under normoxia, HIF-1α protein is rapidly degraded through an oxygen and iron-dependent ubiquitin/proteasome pathway. Under hypoxia, HIF-1α protein is stabilized, translocates into nucleus and heterodimerize with ARNT. This complex associated with the coactivator (CBP/p300) binds hypoxia response element (HRE) in the promoter region of target genes and induces gene expression.
from plasma lipoproteins and the transcription of these genes are regulated by SREBPs.

SREBP is synthesized as precursor and this premature form is bound in the membranes of the endoplasmic reticulum (ER) and nuclear envelope in association with a regulatory membrane protein called SREBP cleavage activation protein (SCAP). In order to regulate transcription, the NH2-terminal domain of SREBPs must be released from the membrane so that it can enter the nucleus. This release is accomplished by a two-step proteolytic cascade regulated by sterols. Under sterol-depleted conditions, SCAP functions as a sensor for cholesterol levels and the SCAP-SREBP complex moves from ER to the Golgi apparatus where SREBPs are cleaved sequentially by site 1 protease and site 2 protease (Figure 2). Then the N-terminal half of the SREBP precursor is released and migrates to the nucleus as the mature form of transcription factor (Wang et al., 1994; Brown and Goldstein, 1997; Sakai et al., 1998). SREBP isoforms (Figure 3) vary in their relative tissue distributions, transcriptional potencies and promoter preferences. SREBP1a and 1c are produced from a single gene (human chromosome 17p11.2) via alternate transcription start sites encoding alternate first exons. The other form of the SREBP, designated SREBP2 is encoded by a separate gene (human chromosome 22q13) (Brown and Goldstein, 1997).

SREBP-1a is a more potent transcription factor than 1c and regulates genes responsible for both cholesterol and fatty acid synthesis. SREBP1c tends
to be more active in regulating genes for fatty acid synthesis and SREBP-2 up-regulates several genes in cholesterol synthesis (Osborne, 2000).

SREBP1a, a member of bHLH/PAS family, can bind the direct repeat sterol regulatory element (SRE) 5'-TCACNCCAC-3' which are present in promoter regions of HMG-CoA reductase, HMG-CoA synthase, LDL receptor genes (Sanchez et al., 1995; Vallett et al., 1996; Dooley et al., 1998). Through the transcriptional regulation of these genes, SREBP1a can regulate the synthesis of cholesterol or its receptor-mediated uptake from plasma lipoproteins.

1.3 The crosstalk between hypoxia pathway and SREBP1a regulated pathway

HIF-1β is a core transcription factor that can dimerize with several related proteins. By using GST pull-down assay and yeast two-hybrid assay, our lab has found that HIF-1β could interact physically with SREBP1a, which regulates cholesterol biosynthesis. This interaction between HIF-1β and SREBP1a suggests the crosstalk between cholesterol biosynthesis and HIF-1β dependent pathway.

Elevated serum cholesterol level is a well-established risk factor for hypertension and coronary heart disease. It’s important to reduce the incidence of cardiovascular disease world wide and drugs synthetically derived HMG-CoA reductase inhibitors such as iovastatin, pravastatin, and simvastatin have been developed for decreasing serum cholesterol levels. While this group of statin
Fig. 2. Model for two-site proteolytic cleavage of membrane SREBPs (Brown, et al., 1997)

Fig. 3. Domain structures of human SREBP-1a and SREBP-2. (Brown, et al., 1997)
drugs can lower the serum cholesterol concentrations, they have serious side
effects. In addition to administration of the statins, some data suggest that the
hypoxia also induces hypocholesterolemic effects. For example, reduced blood
pressure and total serum cholesterol were achieved in hypercholesterolemia
patients exposed to periodic hypoxia in a pressure chamber (Aleshin et al.,
1993). The effects of high altitude exposure on serum cholesterol were studied
in mountaineers. In comparison to the baseline values at low altitude, a
significant reduction in serum cholesterol (27%) was observed after the high
altitude expedition (Ferezou et al., 1988). The hypoxic effects on cholesterol
metabolism were also studied in cultured cells. For instance, it was shown that
hypoxia caused decreased concentration of cholesterol in aortic endothelial cells
(Arai et al., 1996).

Under hypoxia, the genes encoding enzymes involved in glycolysis were up-
regulated to produce more ATP for energy demands. However, cholesterol
synthesis under hypoxia and its molecular mechanism are not well known. It was
reported that the sterol synthesis in cultured rabbit skin fibroblast was repressed
by measurement of the incorporation of $^{14}$C-acetate into sterol (Mukodani et al.,
1990). To investigate the effect of hypoxia on cholesterol biosynthesis in hepatic
cells, we determined the expression of HMG-CoA reductase, the rate-limiting
enzyme in cholesterol biosynthesis pathway, in human hepatoma cells under
hypoxia. We found that hypoxia or hypoxia mimetics, such as CoCl$_2$ could
repress the SREBP1a regulated HMG-CoA reductase expression, and this
repression might be due to the blockage of SREBP1a binding to SRE sequence of HMG-CoA reductase by HIF-1β.
CHAPTER II
MATERIALS AND METHODS

2.1 Cell lines and culture

The human hepatoma line HepG₂ was purchased from ATCC (American Type Culture Collection). The HepG₂ cells were grown in Dulbecco Modified Eagle’s Medium (DMEM) (Sigma) containing 10% (v/v) fetal bovine serum and 100ug/ml antibiotics/antimycotics (Invitrogen). The BPR cell is a mutant line from the murine hepatoma line, which is defective in HIF-1β. The BPR cells were maintained under the same condition as HepG₂ cells. All cells were maintained as a monolayer cultures in a humidified 5% CO₂ atmosphere at 37 °C.

2.2 Plasmids

The pRed-luc and pSREBP1a were obtained from Dr. Timothy Osborne at the University of California, Irvine. The pRed-luc contains the wild-type hamster HMG-CoA reductase promoter from -277 to +20, which was fused to the luciferase coding sequence of the plasmid pGL₂ basic. The pSREBP1a can express SREBP1a (1-490) from the cytomegalovirus promoter.
The plasmid for the recombinant SREBP1a (1-490) protein expression was also from Dr. Timothy Osborne. Briefly, the amino-terminal truncation of SREBP1a was constructed by polymerase chain reaction with the wild type plasmid as template and the polymerase chain reaction product was subcloned into the pRSET expression vector from Invitrogen.

2.3 Transient transfections

HepG2 cells were plated in 12-well plates and then incubated for 16-18 h. At 50-80% confluent state, the dishes were transfected with the indicated plasmids by the lipofectamine method according to the manufacture’s protocols. The luciferase reporter construct pRed-luc (0.5 µg/well) under the control of promoter from HMG-CoA reductase, the expression vector pSREBP1a (0.2 µg/well) were mixed in 60 µl DMEM and then add 60µl DMEM containing 2.5 µl of lipofectamine reagent. 0.2 µg/well of pCMV-βGal encoding β-galactosidase was also added. The total amount of DNA in each transfection was adjusted to the same amount by addition of mock vector plasmid. After 20 min of incubation, 480 µl of DMEM was added to the DNA/Lipofectamine mixture. The cells were washed with phosphate-buffered saline and supplied with 600 µl of the DNA/Lipofectamine mixture. The cells were transfected for 5 h with plasmid, then washed with phosphate buffered saline (PBS) and then grown in DMEM supplemented with 10% fetal bovine serum and 100 µg/ml antibiotic/antimycotic.
The cells were maintained under 21% O₂, 5% CO₂ (Normoxia) or 1% O₂, 5% CO₂ (Hypoxia).

2.4 Enzyme assays

After 24 h, the cells were harvested and lysed by 200 µl of reporter lysis buffer (Promega) and cell debris was removed by centrifugation. Luciferase activities were measured using 10 µl of cell extract and 50 µl of luciferase assay reagent (Promega). β-galactosidase activities were determined according to the manufacture’s protocols (Applied Biosystems). The β-galactosidase activity was used to normalize for any variations in transfection efficiency. Each experiment point was performed in triplicate. The relative luciferase activities (mean +/-S.E., n=3) are shown.

2.5 Northern blot

Total RNA from HepG₂ cells was isolated using TRlzl reagent. The Northern blot was performed as described before (Tian et al., 2003). Twenty micrograms of total RNA from each sample were separated on a 1% agarose/formaldehyde gel and transferred overnight onto a nylon membrane. After UV-cross-linking, membrane was prehybridized for 4 h at 42 °C in prehybridization buffer (6 x SSC, 5x Denhardt's reagent, 0.5% SDS, 100 µg/ml
denatured salmon sperm DNA), and then probed overnight at 42 °C with HMG-CoA reductase cDNA probe labeled with $[\alpha^{32}\text{P}]dCTP$ using Radprime labeling systems (Invitrogen) at $1 \times 10^6$ cpm/ml hybridization buffer (6 x SSC, 0.5% SDS, 100 µg/ml denatured fragmented salmon sperm DNA, 50% formamide). After hybridization, the membrane was washed 3 x 5 min in buffer I (2x SSC, 0.5% SDS), 1x 15 min in buffer II (2x SSC, 0.1% SDS), and then washed with buffer III (0.1% SSC, 0.1% SDS) at 65 °C until the background was low. The wet membrane was exposed at -80 °C overnight using Kodak film. Human HMG-CoA reductase cDNA was obtained using RT-PCR using total RNA from HepG2 cells. The PCR primers were: GATGTCCATGAACATGTTCACC and CGAAGCAGCACATGATCTCCA. The PCR product was inserted to pGEM-T easy vector (Promega). The EcoRI fragment of HMG-CoA reductase was used as template for labeling.

2.6 Mammalian two hybrid assay

pBIND and pACT (Promega) are two hybrid plasmids containing Gal4 DNA binding domain and viral VP16 activation domain, respectively. HIF-1β was fused to Gal4 DNA binding domain as the bait and SREBP1a was fused to VP16 activation domain as the prey. CV1 cells were plated in 12-well plates and then incubated for 16-18 h. At 50-80% confluent state, the dishes were transfected with the pGal4-luc (0.1 µg/well), pACT-HIF-1β (0.5 µg/well) and/or pBIND-
SREBP1a (0.5 µg/well) by the lipofectamine method according to the manufacture’s protocols described as before. After 24 h, the cells were harvested and luciferase activity was determined with a luminometer.

2.7 Pulldown assay

[$^{35}$S]-methionine labeled HIF-1β or luciferase protein were generated by in vitro transcription coupled to translation (TNT translation kit, Promega). SREBP1a (1-490) subcloned into the pRSET expression vector was expressed and purified according to the manufacture’s protocols (Invitrogen). The bacterially expressed and purified SREBP1a was dialyzed against PBS to remove the imidazole. One hundred µl dialyzed SREBP1a was incubated with 10 µl either [$^{35}$S]-methionine labeled HIF-1β or luciferase protein for 2 h at 4°C. The protein complexes were co-precipitated with the Ni-NTA Agarose for 2 h at 4°C. Following the incubation, the beads were washed with PBS containing 0.1% Triton (3x). The proteins were separated by SDS-PAGE and radioactive signals were visualized by autoradiography.

2.8 Protein expression and purification

The baculovirus expression of HIF-1β was performed as described with modification (Chan et al., 1994). The SF-9 cells were grown in Grace’s medium
containing 10% fetal bovine serum and 100 µg/ml antibiotic/antimycotic at room
temperature in a 500 ml spinner flask (Wheaton). Two hundred ml of cells were
infected with baculoviral stocks of the HIF-1β recombinant virus. After 3 days,
the cells were harvested at 800x g for 10 min at 4 °C. After harvest, the cells
were washed with cold PBS once. Then the pellet was resuspended in the 5 ml
of lysis buffer (20 mM Hepes, pH 7.6, 100 mM NaCl, 0.1% NP40, 15% glycerol)
containing 2 mM mecaptoethanol, 50µg/ml aprotinin, and 1 mM
phenylmethylsulfonyl fluoride. The cells were lysed by douncing with pestle A 5
times before being centrifuged at 10,000g for 15 min at 4 °C. The supernatants
were mixed with 1 ml of Ni-NTA-Sepharose (Qiagen) that had been pre-
equilibrated with the same buffer. The suspension was gently mixed at 4 °C for 2
h with rotation. After the resin settled at 4 °C, the solution was passed through,
and the column was washed with 10 ml lysis buffer containing 10 mM imidazole.
To elute the protein that was specifically bound to the resin, we passed 5 ml of
the lysis buffer containing 200 mM imidazole through the column, and the eluate
was collected in 1ml fraction. Western blot analysis of 10% SDS-PAGE was
used to determine the quantity of HIF-1β.

2.9 Western blot

Samples were resolved by 10% SDS-PAGE and then soaked in Towbling
buffer (25 mM Tris.Cl, 200 mM glycine, 0.1% SDS, and 20% methanol) before
the proteins were transferred onto a nitrocellulose membrane using a large Semaphor Transphor Unit (Amersham Pharmacia) for 1 h at constant current 50 mA at room temperature. After transfer, the nitrocellulose membrane was blocked with TBST buffer (25 mM Tris.Cl, pH 7.6, 135 mM NaCl, 2.5 mM KCl, 0.05% Tween 20) containing 5% (w/v) nonfat dry milk overnight at 4 °C. The membrane was incubated with primary antibodies (Anti-HIF-1β (Santa Cruise), 1:1,000) in TBST for 2 h at room temperature. Following this incubation, the membrane was washed with TBST (3x), incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG at 1:2,000 dilution in TBST, washed (3x), and then stabilized substrate of alkaline phosphatase was added.

2.10 Electrophoretic mobility shift assay

Synthetic oligonucleotides were synthesized from Invitrogen. Each oligonucleotide pair was designed such that after hybridization there would be a four-nucleotide single-stranded tail of GATC on each 5’ end. The oligonucleotides are: 5’-GATCGAGAGATGGTGCGGTGCCCGTTCTCC-3’ and 5’-GATCGGAGAACGGGCACCGCACCATCTCTC. Separate oligonucleotides for each strand were annealed as follows. Ten µg of each member of a pair were mixed with its partner in 50 µl solution containing 10 mM Tris.Cl (pH7.8), 1 mM EDTA and 250 mM KCl. The mixture was boiled for 5 min and then incubated at 65 °C for 1 h. The water bath was turned off, and the hybridization
mixtures were allowed to cool slowly to room temperature. An aliquot of the mixture was endlabeled with $\gamma$-32P-ATP and T4 polynucleotide kinase (Promega).

Standard gel shift assays were performed as described (Osborne et al., 1992). DNA binding reaction were set up in a final volume of 20 µl containing 0.5 µg of poly (dI.C), 5%(w/v) Ficoll, 25 mM Tris.Cl (pH7.9), 0.5 mM EDTA, 50 mM KCl, 5 mM MgCl2. After incubation for 30 min on ice, the samples were loaded onto a 5% polyacrylamide gel (29:1, acrylamide:bis) containing 5% glycerol in 0.5x TBE. The gels were subjected to electrophoresis at 4°C for 30 min prior to loading the samples, after which they were subjected to electrophoresis for 4-5 h at 200v at 4°C. The gels were dried onto paper followed by autoradiography.

2.11 In vitro transcription

By using HeLaScribe® Nuclear Extract in vitro Transcription Kit, in vitro transcription was performed according to the manufacture’s instruction (Promega). Briefly, the plasmid construct pRed-luc containing a reporter gene under the control of HMG-CoA reductase SRE segment was linearized with Xbal. One hundred ng linearized SRE-driven template was incubated with 0.2 µg SREBP1a protein in the presence of Hela nuclear extract which contained Pol II and other factors for the basic transcriptional machinery. The RNA products were obtained with phenol-chloroform extraction and analyzed with denaturing polyacrylamide gel (29:1, acrylamide:bis) containing 7M urea in 1x TBE. The gels
were subjected to electrophoresis for 4 h at 200v at room temperature. The gels were dried onto paper followed by autoradiography.
CHAPTER III
RESULTS

3.1 Physical interaction between HIF-1β and SREBP1a

To determine the interaction between SREBP1a and HIF-1β, a mammalian two-hybrid assay was performed. Co-expression of pBIND-SREBP1a and pACT-HIF-1β in CV-1 cells markedly activated luciferase reporter gene, indicating SREBP1a associates with HIF-1β (Figure 4A).

This interaction was also confirmed by pull-down assay (Figure 4B). The HIF-1β was pulled down by the Ni-NTA resin bound with SREBP1a (lanes 3 and 4), indicating that SREBP1a specifically interacted with HIF-1β.

3.2 Hypoxia suppresses HMG-CoA reductase gene expression

Plasmids containing luciferase reporter gene driven by HMG-CoA reductase and SREBP1a were cotransfected into HepG2 cells. The cells were then kept in normoxia (5% CO₂, 21% O₂), hypoxia (5% CO₂, 1% O₂) or normoxia with indicated concentration of CoCl₂ for 24 h. The cells were harvested and luciferase activity was determined with a luminometer.
Fig. 4. Interaction between HIF-1β and SREBP1a. (A) Mammalian two hybrid assay. CV-1 cells were maintained in DMEM containing 10 % FBS. The cells were co-transfected with the indicated plasmid constructs. After 24 h, the cells were harvested and luciferase activity was determined with a luminometer. (B) In vitro pulldown assay. The bacterially expressed and purified SREBP1a was incubated with either $[^{35}\text{S}]$-methionine labeled HIF-1β or luciferase protein. The protein complexes were co-precipitated with the Ni-NTA Agarose and the proteins were separated by SDS-PAGE followed by autoradiography. Lanes 1, 2 were 1/10 of the input of HIF-1β and luciferase proteins, respectively. Lane 5, molecular mass marker; lane 6, affinity purified SREBP1a protein (Coomassie blue stain).
The result shows that hypoxia or hypoxia mimetic, CoCl₂ can repress SRE-driven luciferase reporter gene expression in HepG₂ cells (Figure 5A). The repression of luciferase activity by CoCl₂ is dose dependent (lane 2, 3, 4, 5). And the hypoxia treatment significantly decreases the luciferase activity. (Lane 2, 7).

The effect of hypoxia on HMG-CoA reductase SRE driven luciferase reporter gene expression was also investigated in mouse primary cells. Following co-transfection with pRed-luc and pSREBP1a, the mouse primary cells were kept under normoxia or hypoxia and the luciferase activity was determined after 16 h. The luciferase gene expression was repressed significantly under hypoxia (Figure 5B).

To determine the effect of hypoxia on the HMG-CoA reductase expression level, HepG₂ cells were grown in DMEM containing lipid-depleted FBS under normoxia (5% CO₂, 21% O₂) or hypoxia (5% CO₂, 1% O₂) at different time-point. Total RNA was prepared and Northern Blot was performed (Figure 6A.). The data shows that HMG-CoA reductase mRNA expression is suppressed under hypoxia or by CoCl₂ treatment. The repression by hypoxia in HepG₂ cells or mouse primary cells was further confirmed by real-time PCR (Figure 6B, C.).
Fig. 5. HMG-CoA reductase SRE driven reporter gene expression under hypoxia. A. HepG2 cells were grown in DMEM containing 10% FBS and then transiently transfected with the indicated plasmid constructs. The cells were then maintained under normoxia (5% CO₂, 21% O₂), hypoxia (5% CO₂, 1% O₂) or normoxia with CoCl₂ for 24 h. The cells were harvested and luciferase activity was determined with a luminometer. The asterisks represent values significant different from normoxia (P<0.05). B. Mouse primary hepatocytes were transiently transfected with pSREBP1a and pRed-luc. The cells were then maintained under normoxia (5% CO₂, 21% O₂) or hypoxia (5% CO₂, 1% O₂) for 16 h. The cells were harvested and luciferase activity was determined with a luminometer. The asterisk represents a value that is significant different from normoxia (P<0.05)
Fig. 6. Regulation of HMG-CoA reductase gene expression by hypoxia or CoCl$_2$. A. HepG$_2$ cells were kept under hypoxia (1% O$_2$, 5% CO$_2$), or under normoxia (21% O$_2$, 5% CO$_2$) in the presence of 100 µM CoCl$_2$. Total RNA was prepared at indicated time after treatment. Northern blot analysis was performed. Even loading on the lanes was monitored by staining the gel with ethidium bromide.
Fig. 6. Continued. B. HepG2 cells were kept under normoxia (5% CO₂, 21% O₂) or hypoxia (5% CO₂, 1% O₂). mRNA levels were analyzed by real-time PCR. β-Actin RNA was used as internal standard. The asterisk represents a value that is significant different from normoxia (* 0.05<P<0.1, **P<0.05) C. Mouse primary hepatocytes were grown in DMEM containing lipid-depleted FBS under normoxia (5% CO₂, 21% O₂) or hypoxia (5% CO₂, 1% O₂) for 6 h. mRNA
levels were analyzed by real-time PCR. β-Actin RNA was used as internal standard. The asterisk represents a value that is significant different from normoxia (P<0.05)

3.3 HIF-1β blocks the SREBP1a binding to the SRE in the promoter region of HMG-CoA reductase

Hypoxia can repress the expression of HMG-CoA reductase. Based on the interaction between SREBP1a and HIF-1β, we hypothesize that this repression is caused by the blockage of SREBP1a binding to SRE by HIF-1β. To determine the effect of HIF-1β on SREBP1a binding to the SRE sequence in vitro, electrophoretic mobility shift assay (EMSA) was performed using in vitro expressed SREBP1a. The HIF-1β was obtained by baculovirus expression and the quality of the protein was determined by SDS-PAGE and Western blot assay (Figure 7B.). The result shows that HIF-1β can block the SREBP1a binding to the SRE in the promoter region of HMG-CoA reductase gene (Figure 7A. lane 1, 3, 4.).

3.4 Ectopic expression of HIF-1β represses SRE driven luciferase reporter gene expression in BPR cells

To detect the effects of HIF-1β on the SRE driven luciferase reporter gene expression, BPR cells which are HIF-1β deficient were co-transfected with pRed-luc, pSREBP1a and pHIF-1β. The data showed that the reintroduction of HIF-1β
repressed the HMG-CoA reductase SRE driven reporter gene expression (Figure 8, lane 2, 3, 4).

Fig. 7. HIF-1β suppresses SREBP1a binding to SRE site. A. Electrophoretic mobility shift assay (EMSA) was performed using in vitro expressed SREBP1a. Lane 1 shows the SREBP1a binding to SRE, lanes 3 and 4 show the suppression of SREBP1a binding to SRE in the presence of HIF-1β. Lanes 5, 6 and 7 is the same as lanes 1, 3 and 4, respectively except that no DNA template was in the in vitro translation. B. Baculovirus expression and affinity-purified HIF-1β. Crude: crude lysate. FT: flow through. W: wash. E1: fraction 1 of eluate. E2: fraction 2 of eluate.
Fig. 8. HIF-1β suppresses SRE-driven reporter gene expression. BPR cells (HIF1β-/-) were grown in DMEM containing 10% FBS and transiently co-transfected with the indicated plasmid constructs. After 18 h, cells were harvested and luciferase activity was determined with a luminometer. The asterisk represents a value that is significant different from lane 2 (P<0.05).
3.5 *In vitro transcription*

To further determine the effect of HIF-1β on expression of SREBP1a-regulated gene, in vitro transcription will be performed. In vitro transcription is a useful technique for the study of the regulation of gene transcription. By using HeLaScribe® Nuclear Extract in vitro Transcription Kit from Promega, some pilot experiments have been done. RNA yield from in vitro transcription increased significantly in presence of transcriptional factor (SREBP1a) (Figure 9, lanes 3 and 4). In the future study, the HIF-1β protein will be added and allowed to incubate with SREBP-1a and the chromatin template during in vitro transcription.

3.6 *Hypoxia or CoCl2 induces DEC1 gene expression*

Our result suggests that hypoxic repression of HMG-CoA reductase expression is mediated by interaction of SREBP1a and HIF-1β. Also we speculate that hypoxia induced transcriptional repressor might be involved in this repression. In this study, the expression of hypoxia-regulated DEC1, which is a transcriptional repressor, was determined by using real-time PCR. The data showed that DEC1 expression was enhanced after 3-6 h under hypoxia or CoCl2 treatment in HepG2 cells (Figure 10).
Fig. 9. In vitro transcription. SRE-driven template plasmid was incubated with SREBP1a protein in the presence of Hela nuclear extract. The RNA products were extracted and analyzed with denaturing polyacrylamide gel. The arrow shows the RNA product incorporated with $\alpha^{32}$P-UTP.

Lane 1 shows molecular weight marker
Lane 2: Positive control
Lane 3: SRE driven template
Lane 4: SRE driven template + SREBP1a
Fig. 10. Regulation of DEC1 gene expression by hypoxia or CoCl2. HepG2 cells were kept under hypoxia (1% O2, 5% CO2), or under normoxia (21% O2, 5% CO2) in the presence of 100 µM CoCl2. Total RNA was prepared at indicated time after treatment. The cDNA was obtained by reverse transcription and real-time PCR was performed.
CHAPTER IV
CONCLUSION AND DISCUSSION

Our results show that the hypoxia can repress expression of HMG-CoA reductase, which is the rate-limiting enzyme in the cholesterol biosynthesis pathway. Transient transfection assays shows that hypoxia or hypoxia mimetic, CoCl₂ can repress SRE-driven luciferase reporter gene expression. In addition, HMG-CoA reductase mRNA levels are repressed under hypoxia and this suppression is observed most significantly between 3-6 h after hypoxia treatment (Figure 6B). Based on results of gel shift assays, we suggest that this suppression is caused by inhibition of the SREBP1α binding to the SRE sequence by HIF-1β, which can physically interact with SREBP1α.

Under hypoxia, HMG-CoA reductase mRNA levels significantly decreased in 3-6 h. However, mRNA levels were back to normal after 24 h (Figure 6B). This might be due to a negative feedback mechanism. The transcription of HMG-CoA reductase is tightly controlled by the mature form of SREBP1α. Under cholesterol-depleted conditions, the premature form of SREBP1α is processed through a two-step cleavage. The N-terminal half of the SREBP1α is released and enters the nucleus as the mature form of transcriptional factor. After 3-6 h of hypoxia treatment, the HMG-CoA reductase expression is repressed and the cholesterol levels are decreased. Decreased cholesterol levels activate the maturation process of SREBP1α, this will increase the HMG-CoA reductase.
expression. The microarray studies show several hundred of genes were down-regulated under hypoxia in mouse fibroblasts (Vengellur et al., 2003; Greijer et al., 2005). However, the repression of HMG-CoA reductase was not observed. In these studies, the cells were treated under hypoxia for at least 24 h. No pronounced effect on HMG-CoA reductase expression after long period of exposure to hypoxia might be due to this feedback mechanism.

Hypoxia represses the expression of the HMG-CoA reductase and this repression might be mediated by interactions between SREBP1a and HIF-1β. HIF-1β is a transcriptional factor which can also dimerize with the Ah receptor (AhR) in response to AhR ligand such as TCDD. Therefore, the TCDD might also regulate the expression of HMG-CoA reductase through a similar pathway. Some preliminary studies in our lab have shown some hypocholesterolemic effects of TCDD in C3H/HeNCr mice. For example, the mice were fed with “Western diet”, which is high in fat. TCDD treatment caused significant suppression of serum cholesterol. To determine if this hypocholesterolemic effect induced by TCDD is mediated by the repression of HMG-CoA reductase expression, the effects of TCDD on mRNA levels in cells will be further determined.

Our data show that HIF-1β can interact physically with SREBP1a. Results of the gel shift assay suggest that this interaction lead to blockage of the SREBP1a binding to the SRE sequence in vitro. To determine if SREBP1a binding to the SRE sequence of the HMG-CoA reductase promoter region is repressed in vivo
under hypoxia, the chromatin immunoprecipitation (ChIP) assay will be performed.

In addition to this HIF-1β mediated transcriptional repression, other molecular mechanisms may also be involved. Some studies showed that the hypoxia regulated transcriptional repressors such as Bach-1 might be involved in transcriptional repression by hypoxia. For example, hypoxia induced suppression of heme oxygenase 1 (HO-1) has been associated with induction of Bach-1, a heme-regulated transcriptional repressor (Kitamuro et al., 2003). In ovarian carcinoma cells, hypoxia induce down-regulation of E-cadherin via up-regulation of the transcriptional repressor SNAIL (Imai et al., 2003).

The HIF-1α regulated gene DEC1/Stra13, a novel bHLH transcriptional factor (BHLHB), represses PPARγ2 promoter activation and functions as an effector of hypoxia-mediated inhibition of adipogenesis (Yun et al., 2002). DEC1/Stra13 mRNA expression is induced under hypoxia or CoCl2 treatment in 293T, Hela and ATDC cells (Miyazaki et al., 2002) and the functional hypoxia response element (HRE) has been identified in DEC1/Stra13 promoter region. Our results suggest that DEC1 expression is also induced by hypoxia in HepG2 cells (Figure 10.). The DEC1/Stra13 can function as a transcription repressor through interaction with TATA binding protein (TBP) or RNA polymerase II complex thus interacting with the basal transcriptional machinery (Boudjelal et al., 1997). The DEC1/Stra13 can also repress transcription via histone deacetylase (HDAC)-dependent pathway (Sun and Taneja, 2000). The Effect of
induced DEC1 by hypoxia on HMG-CoA reductase expression needs to be further studied.

Also, it was reported that the α–fetoprotein (AFP) expression in hepatoma cells could be repressed under hypoxia. Under hypoxia, the negative factor 2 (NC2) α or β protein is stabilized, therefore block the transcription by association with DNA-bound TFIID and inhibit the preinitiation complex assembly (PIC) formation (Denko et al., 2003). So the role of NC2 α/β in repression of HMG-CoA reductase expression under hypoxia will be explored in the future study.
REFERENCES


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