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Expression of hypoxia-regulated genes and glycometabolic genes in placenta from patients with intrahepatic cholestasis of pregnancy

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ABSTRACT

Introduction: Intrahepatic cholestasis of pregnancy (ICP) is a liver disorder unique to pregnancy that is associated with increased rates of fetal distress and demise. While acute hypoxia is believed to cause the pathophysiology of ICP, direct molecular evidence for this is lacking. Here, we analyzed expression of three hypoxia-regulated genes and several of their downstream target genes involved in glucose metabolism in placenta.

Methods: Placental tissue was collected from 20 women with normal pregnancies and 20 women with ICP. RNA and protein levels of hypoxia inducible transcription factors -1α (*HIF-1α*), development and DNA protein damage response 1 (*REDD1*), mammalian target of rapamycin (*mTOR*), glucose transporter type 1 (*GLUT1*), phosphoglycerate kinase1 (*PGK1*) and lacticdehydrogenase (*LDHA*) in placental tissue were measured by reverse transcriptase real time PCR and Western Blot. Proteins were also located by immunohistochemistry.

Results: Transcript levels were similar for all genes between the two types of placental tissue. In contrast, all protein levels except that of mTOR were significantly higher in placentas from ICP patients than the controls ($P < 0.05$). All proteins localized to the cytotrophoblast and syncytiotrophoblast.

Discussion: The placenta from ICP patients is more vulnerable to acute hypoxia and ischemia reperfusion injury. In response to hypoxia stress and oxidative damage in ICP, the placenta activates HIF-1α and REDD1, which in turn may up-regulates glucose transport and anaerobic glycolysis.

Conclusions: HIF-1α, REDD1 and mTOR may play a significant role in the reaction to hypoxia and oxidative stress and regulate glucose metabolism in the placenta of ICP patients.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver disorder. The prevalence of ICP varies significantly among nations and ethnic groups, affecting 1–5% of pregnancies in China [1]. Characterized by elevated bile acids in maternal blood, this condition is potentially lethal to the fetus and is associated with fetal distress and fetal demise [2], the mechanism by which remains unclear. Bile acids may cause vasoconstriction of placental veins [3] and impair placental morphology and functionality [4]. In placentas from cholestatic pregnant rats, the bile acid was shown to impair the antioxidant system and induce oxidative damage [5]. Oxidative damage may also involve in the pathophysiology of ICP [6–7]. Autopsy of stillbirths and study on umbilical cord blood of fetuses of ICP patients suggested that the placenta in women with ICP is affected by acute hypoxia [8–9]. In fact, acute hypoxia may explain the strong association between uterine contractions, which naturally act as an anoxic challenge to the fetus, and intrauterine fetal death in women with ICP [2]. Therefore, we hypothesize that in ICP patients, the pathological environment makes the placenta more vulnerable to acute hypoxia and ischemia reperfusion injury. The impairment of placental function may contribute to subsequent adverse fetal outcomes.

Placentas could adaptively respond to hypoxia by upregulating the levels of genes involved in glucose transport and glycolysis, promoting energy production under oxygen insufficiency [10]. Hypoxia is the primary regulator of hypoxia-inducible transcription factor 1α (*HIF-1α*), a key part of the HIF-1 heterodimer, which in turn may also induce transcription of genes encoding glucose transporter type 1 (*GLUT1*), phosphoglycerate kinase1 (*PGK1*) and lacticdehydrogenase (*LDHA*) in a variety of tissues [11–12].

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Consistent with this possibility, our previous study indicated *HIF-1α* was a sensitive indicator of placenta hypoxia in ICP patients [13]. This suggests the possibility that *HIF-1α* plays a central role in the placental response to hypoxia in ICP. *HIF-1* up-regulates development and DNA protein damage response 1 (*REDD1*), a newly-discovered *HIF-1* receptor, which is an essential regulator of mammalian target of rapamycin (*mTOR*) activity [14]. The mTOR signaling pathway has been shown to regulate cell growth through interaction between nutrients and glucose and is significant in cell hypoxia response [15].

Previous studies indicated adaptive alterations in the *HIF-1α, REDD1* and mTOR expression and consequently in glycolmetabolism under hypoxia condition [13–15]. To explore placental hypoxia in ICP and investigate molecules involved in the placental response to this stress, we examined the mRNA and protein expression of the hypoxia-regulated genes *HIF-1α, REDD1* and *mTOR*, as well as of downstream genes involved in glucose metabolism *(GLUTI, PGK1, LDHA*), in placentas from women with ICP and from women with normal pregnancies.

2. Methods

2.1. Patients and controls

This study involved 20 women with ICP pregnancies and 20 women with normal pregnancies who underwent elective cesarean deliveries in the Department of Obstetrics and Gynecology of West China Second University Hospital, Sichuan University. The study was approved by the Ethics Committee of West China Second University Hospital, and all patients gave informed consent. Clinical characteristics of 40 women were collected (Table 1) [16]. ICP was diagnosed on the basis of severe pruritus, absence of skin rash during the second half of gestation, and elevated levels of serum alanine transaminase (ALT), aspartate transaminase (AST) and serum total biliary acid (TBA) (>10 μmol/L). Patients were excluded if their pruritus could be attributed to causes other than ICP [6] or if they had gallstones, cholecystitis or liver cirrhosis. All patients received lab tests to exclude the infection of hepatitis virus, Epstein Barr virus and cytomegalovirus.

2.2. Tissue collection

Placentas were collected immediately after cesarean delivery and before labor onset. Villous samples were taken systematically from eight lobules of each placenta. Tissue samples were briefly rinsed in ice-cold phosphate-buffered saline (PBS) and transferred from the hospital to the laboratory in cold PBS within 20 min of collection. For tissue collection, a circle with a 10 cm radius from the umbilical cord insertion site was identified and 8 samples (approximately 0.5 g each) were collected from equally spaced points around the circumference of this circle. A cylindrical sample of placental tissue with a diameter of 5 cm was obtained by cutting across the placenta from the chorionic plate down to the basal plate. Then the tissue was transferred within 10 min of collection from the hospital to the laboratory in cold 0.9% saline solution. The cylinder was sectioned into blocks 10-mm thick, which were immersed in formalin at room temperature to allow analysis of morphology. Placental villous samples for RNA extraction and protein analysis were snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Quantitative real-time RT-PCR

Placental samples were first pulverized using a sterile hand-held homogenizer. Total RNA (about 5 μg) was extracted from around 5 mg sample using TRIzolreagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and dissolved in 20 μl of RNase-free water. The RNA was reverse-transcribed with PrimeScript® RT reagent Kit with gDNA Erase (TaKaRa, Dalian) using random hexamers.

Table 1
Clinical characteristics of women with normal pregnancies or ICP.

Characteristic	Normal ($n = 20$)	ICP ($n = 20$)	*P* value
Maternal age, year	33.3 (4.0)	30.25 (4.7)	0.17
Gravidity	3.8 (3.1)	2.1 (1.4)	0.16
Parity	1.4 (0.7)	1.3 (0.4)	0.52
Gestational age, week	38.4 (0.9)	37.49 (0.4)	0.09
Birth weight, g	3252.5 (443.2)	3001.4 (210.3)	0.18
Placental weight, g	559.89 (96.48)	553.44 (85.58)	0.84
Placental index[a]	0.16 (0.02)	0.17 (0.04)	0.49
Pre-pregnant BMI	21.32 (2.80)	20.60 (2.43)	0.43

Values are mean ± SD.
[a] Placental index: placental weight divided by birth weight.

Specific primers were then used to amplify six genes (Table 2): *HIF-1α, REDD1, mTOR, GLUT1, PGK1* and *LDHA*. These amplifications were carried out in a final volume of 20 μl containing 1 μl of template cDNA, 2 × Brilliant SYBR®Green QPCR Master Mix (with 2.5 mM $MgCl_2$), 300 nM of each primer and 30 μM Rox passive reference dye. After initial denaturation at 94 °C for 2 min, samples were subjected to 40 cycles of denaturation at 94 °C for 20 s, annealing at a gene-specific temperature (Table 2) for 30 s, and elongation at 72 °C for 40 s. Amplification and elongation steps were followed by melt curve analysis in which the temperature was increased from 55 to 95 °C at 0.2 °C/sec. Transcript levels for the genes of interest were normalized to those of the β-actin housekeeping gene assayed in parallel.

2.4. Western blotting

Placental samples (100 mg) were homogenized in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.1% SDS, 1 mM dithiothreitol, and 1 × Complete Protease Inhibitor Cocktail (Roche). Homogenates were centrifuged at 12,000 *g* for 10 min at 4 °C and the supernatants were collected. Total protein concentration in the supernatants was determined using the Bio-Rad assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes at 60 V/250 mA for 70 min. Membranes were blocked for 2 h at room temperature in Tris-buffered saline containing 5% skim milk and 0.1% Tween 20. Then blots were incubated overnight at 4 °C with primary antibodies (Table 3). Levels of the protein of interest were normalized to levels of β-actin as control.

Membranes were washed three times with washing buffer, and primary antibody binding was visualized using the Envision™ Detection Kit (Gene Tech, Shanghai, China). Relative band densities were quantitated by densitometry using Quantity One software (Bio-Rad).

2.5. Immunohistochemistry

Placenta villous tissues were fixed in PBS with 10% formaldehyde for 24 h at 4 °C, dehydrated in a graded series of ethanol solutions and embedded in paraffin (Leica, Germany) at 52–58 °C. Serial sections (4–5 μm) were deparaffinized with xylene and rehydrated in a graded series of ethanol solutions (5 min each step). Expression of target proteins in the sections was then measured by immunohistochemistry using the following procedure. Sections were subjected to an antigen retrieval method using citrate buffer (pH 6.0) at 95 °C for 40 min. After endogenous peroxidase was inactivated by soaking in 3% hydrogen peroxide, sections were incubated with blocking serum containing the following primary antibodies (Table 3) for 30 min at room temperature: *HIF-1α* (1:100), *REDD1* (1:200), *mTOR* (1:50), *GLUT1* (1:200), *PGK1* (1:100) and *LDHA* (1:100). Antibody binding was visualized using the Histofine Simple Stain PO (M) kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Peroxidase activity was detected by incubating sections with DAB substrate, producing a brown stain. Sections were counterstained with hematoxylin. Finally, stained slides were examined on a light microscope (Olympus, Tokyo, Japan). As negative controls, tissue sections were incubated in parallel with isotype IgG at the same concentration as the primary antibody or with only secondary antibody without primary antibody.

2.6. Statistical analyses

Data were analyzed using SPSS for Windows 11.0 (IBM, Chicago, USA). Levels of *HIF-1α, REDD1, mTOR, GLUT1, PGK1* and *LDHA* mRNA and protein were compared between placental samples from ICP pregnancies and from normal pregnancies

Table 2
Primers and amplification conditions for real-time RT-PCR.

Primer name	Primer sequence	Amplicon (bp)	Annealing temp (°C)
HIF-1α F[a]	TGCTGATTTGTGAACCCATTCC	186	46
HIF-1α R[b]	CCAAAGCATGATAATATTCATA		
REDD1 F	GAGCCTGGAGAGCTCGGACT	154	56
REDD1 R	CTGCATCAGGTTGGCACACA		
mTOR F	ATGCTGTCCCTGGTCCTTAT	176	56
mTOR R	GTCAGAGAGTGGCCTTCAAA		
GLUT1 F	GGCATCAACGCTGTCTTCTAT	126	56
GLUT1 R	CACAAACAGCGACACGACAGT		
PGK1 F	GCCACTTGCTGTGCCAAATG	102	56
PGK1 R	CCCAGGAAGGACTTTACCTT		
LDHA F	CCAGCGTAACGTGAACATCTT	195	56
LDHA R	CCCATTAGGTAACGGAATCG		
β-actin[c] *F*	GAAGATCAAGATCATTGCTCCT	111	54
β-actin R	TACTCCTGCTTGCTGATCCACA		

[a] F, forward primer.
[b] R: reverse primer.
[c] β-*actin* (beta-actin, house-keeping gene).