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Dysregulated Hepatic Expression of Glucose Transporter Type-1, Toll-Like Receptor 4, and Nuclear Factor Kappa B in Estrogen-Induced Cholestasis Pregnant Rats with Placental Ischemia-Reperfusion Stress

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Abstract

Objective: This study aimed at investigating the expression of nuclear factor kappa B (NF-κB) and mammalian target of rapamycin (mTOR) related signal pathways in liver tissues of intrahepatic cholestasis of pregnancy animal models.

Methods: Estrogen (EE)-induced cholestasis and a placental ischemia-reperfusion (IR) model were established in pregnant rats. All pregnant rats were divided into four groups by random number table: EE-IR group ($n=6$), EE-sham group ($n=6$), control-IR group ($n=6$) and control-sham group ($n=6$). Liver expression of mTOR, its upstream regulator DNA damage response-1 (REDD1), and downstream factor glucose transporter type-1 (GLUT1), accompanied by NF-κB (p65 is the most important component), its activator toll-like receptor 4 (TLR4), and inhibitor IκBα, were detected by western blot analysis and real-time polymerase chain reaction. The intergroup comparisons were performed with a one-way analysis of variance, the comparisons among groups were analyzed with the nonparametric Kruskal-Wallis test.

Results: Giving pregnant rats EE alone reduced the hepatic expression of IκBα (0.72 ± 0.20 *vs.* 1.01 ± 0.07, $P=0.008$). Meanwhile, giving pregnant rats placental IR alone increased liver levels of REDD1 (3.24 ± 0.98 *vs.* 1.06 ± 0.24, $P=0.025$), GLUT1 (2.37 ± 0.82 *vs.* 1.09 ± 0.10, $P=0.039$), TLR4 (2.12 ± 0.29 *vs.* 1.20 ± 0.28, $P=0.010$), and p65 (2.09 ± 0.85 *vs.* 1.04 ± 0.06, $P=0.023$), and decreased hepatic mTOR (0.50 ± 0.07 *vs.* 1.01 ± 0.03, $P=0.001$) and IκBα (0.61 ± 0.08 *vs.* 1.01 ± 0.07, $P=0.014$) expression. Subjecting EE-treated rats to placental IR did not further alter liver levels of GLUT1 (2.02 ± 0.45 *vs.* 1.79 ± 0.39, $P=0.240$), TLR4 (2.10 ± 0.74 *vs.* 1.60 ± 0.36, $P=0.129$), or p65 (2.41 ± 0.83 *vs.* 1.65 ± 0.46, $P=0.145$), whereas it did decrease hepatic mTOR (0.42 ± 0.09 *vs.* 0.90 ± 0.14, $P=0.008$) and IκBα (0.43 ± 0.09 *vs.* 0.72 ± 0.20, $P=0.004$) expression and enhance REDD1 expression (4.46 ± 0.65 *vs.* 2.05 ± 0.47, $P=0.009$). Placental IR stress did impact the hepatic expression of REDD1-mTOR-GLUT1 and TLR4/NF-κB/IκBα in pregnant rats.

Conclusion: Placental IR-induced hepatic GLUT1, TLR4, and p65 alternation, which responded efficiently in control rats, were impaired in EE-induced ICP rats.

Keywords: Cholestasis, intrahepatic; Liver; mTOR; Pregnancy

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is the most common liver disorder during pregnancy. The incidence of ICP, reported to be 0.1% to 4.0%, varies with different geographic regions and ethnicities.[1,2] ICP is complicated with unpredictable fetal anoxia and even intrauterine fetal death.[2] The pathogenesis of ICP is not fully understood. ICP is characterized by increased total bile acid (TBA) levels, alanine transaminase (ALT), and aspartate transaminase (AST) in the maternal blood. However, clinical research indicates that fetal hypoxia and/or intrauterine fetal death mostly occur in the late trimester of pregnancy or early labor stage with uterine contractions.[3,4] This clinical phenomenon implies the possible role of uterine-placental-fetal ischemia-reperfusion (IR) in adverse fetal outcomes in ICP.

Immune disorders contribute to the onset of ICP.[3,5,6] Studies of placental gene expression profiles in ICP have shown that the immunity-related genes were predominately

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changed in ICP patients.[7] Evidence suggesting that the mechanisms of ursodeoxycholic acid in improving cholestasis were attributed in part to immune modula-tion.[8] Moreover, it had been widely accepted that the pathomechanisms of ICP closely correlate with the liver and placenta, the two most vital organs related to immunoregulation during pregnancy.[1–3] The immunologic connections underlying the liver and placenta in ICP remain poorly understood.

Nuclear factor kappa B (NF-κB) plays a central role in immune-related pathological processes in both the placenta and liver.[6,9] The NF-κB family are critical regulators of the immune process during pregnancy and control the T helper cell-1 and T helper cell-2.[6] Additionally, the activation of NF-κB was tightly controlled by toll-like receptors 4 (TLR4).[10] The inhibitor of nuclear factorkappa B (IκB) serves as the master negative-regulatory element of the NF-κB signaling pathway.[11] Studies have demonstrated that the NF-κB expression in ICP placentas and taurocholate acid-treated HTR8/SVneo cells were significantly upregulated compared with those in normal pregnancy and non-treated HTR8/SVneo cells.[6] It has also been reported that the expression of TLR4 and NF-κB (p65 is the most important component) were markedly increased in bile duct ligation rats compared with sham rats.[9] All that evidence implies the possible role of NF-κB regulation in the pathophysiology of ICP. However, the expression of TLR4/NF-κB/IκB in livers from ICP has not been investigated.

Accumulating evidence has shown that immune function and metabolism were closely linked by mammalian target of rapamycin (mTOR),[12] with mTOR playing a critical role in regulating immune activities by integrating cues from the microenvironment.[13,14] Additionally, glucose transporter type 1 (GLUT1), a downstream factor of mTOR,[15,16] serves as a basal glucose uptake factor in placental trophoblast cells and is essential for immune cell activation.[17,18] Studies indicated that mTOR, GLUT1, and their upstream regulator regulated the development of DNA damage response 1 (REDD1)[13,15,16] were dysregulated in placentas from both ICP patients and estrogen (EE)-induced ICP rats.[19,20] How immune and glycometabolism-related factors are expressed in liver tissues of ICP remains to be explored.

The aim of this study was to investigate the expression of REDD1-mTOR-GLUT1 signal pathway and TLR4/NF-κB/IκB signal pathway in liver tissues of EE-induced ICP rats and its placental IR models. Our results will help to reveal the hepatic expression of immune- and metabolism-related factors in EE-induced ICP rats.

Material and methods

Animals

This study was reviewed and approved by the Ethical Committee of the West China Second University Hospital, Sichuan University (Medical research 2018 application 02). Adult female Sprague-Dawley rats weighing 250–300 g were mated with male Sprague-Dawley rats with a 1:1 ratio. The vaginal plugs were checked every morning to confirm the pregnancy. The day a vaginal plug was found would be recorded as the first day of gestation. Pregnant rats were maintained in a room with a 12-h photocycle of light/dark and controlled temperature/humidity (18 °C–20 °C/ 50%–70%). Animals were housed with a standard water and diet ad libitum. All the rats received humane care according to the criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Treatment and control groups

All pregnant rats were divided into four groups by random number table (EE-IR group, EE-sham group, Control-IR group, and Control-sham group), each group included 6 rats, on day 14 of gestation. The EE-IR group was subcutaneously administered with ethinyl estradiol (EE 5 mg/kg body weight in propylene glycol as solvent; Sigma, Shanghai, China) for 5 days and began on day 14 of gestation[21] with performance of feto-placental IR[22] (as described in previous study[20]) on day 19 of gestation. The utero-ovarian artery was bilaterally closed off for 20 min with soft polyvinyl tubing covered forceps, and reopened for 30 min to allow reperfusion. Sham-operated animals were subjected to the same procedure except for closing off the utero-ovarian artery.[22] The EE-sham group was administered with EE, as mentioned above, except for mock IR operation on day 19 of gestation. The control-IR group received only propylene glycol (5 mg/kg body weight) for 5 days started beginning on day 14 of gestation, with performance of feto-placental IR on day 19 of gestation. The control-sham group received propylene glycol as mentioned above except for being subjected to mock IR on day 19 of gestation. Pregnant rats in this study were anesthetized with a single intraperitoneal dose of chloral hydrate (2.5 mg/kg body weight). Additionally, in pregnant rats with fetoplacental IR, we administered 1.0% isoflurane (carried by pure O_2 at a flow rate of 1.0 L/min) to maintain steady anesthesia. Liver tissues were sampled after the IR or sham operation. Blood samples were collected using ethylene diamine tetraacetic acid (EDTA)-containing tubes and were centrifuged for 15 min at 1000 ×*g* at 2 °C–8 °C within 30 min. ALT, AST, and TBA were detected using a fully automated biochemical analyzer (Siemens Electrical Apparatus Ltd., Munich, Germany). All animals were sacrificed by exsanguination after collection of samples.

Real-time polymerase chain reaction (PCR)

Total RNA was prepared from liver tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. mRNA was reverse transcribed into cDNA using the Revert Aid Frist Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Cambridge, MA, USA). The following primers were used: REDD1, forward 5'-GAGCCTGGAGAGCTCGGACT-3', reverse 5'-CTGCATCAGGTTGGCACACA-3', probe 5'-CTGTTGCTGCTGTCCAGGGAC-3'; mTOR, forward 5'-ATGCTGTCCCTGGTCCTTAT-3', reverse 5'-GTCAGAGAGTGGCCTTCAAA-3', probe 5'-ACCCTCCCTGAGGCCAGCGA-3'; GLUT1, forward 5'-GGCATCAACGCTGTCTTCTAT-3', reverse 5'-CACAAACAGCGACACGACAGT-3', probe 5'-CAGCAGCCTGTGTATGCCACCA3'; p65 (the most important component of NF-κB),

forward 5'-TCTGGCGCAGAAGTTAGGT-3', reverse 5'-CCAGAGACCTCATAGTTGT-3', probe 5'-GGACTC AGCCGGAAG GCATT-3'; TLR4, forward 5'-GAAGCTA TAGCTTCACCAAT-3', reverse 5'-GATAGGGTTTCC TGTCAGT-3', probe 5'-CACACCTGGATAAATCCAG CCAC-3'; IκBα, forward 5'-AACCTGCAGCAGACTC-CACT-3', reverse 5'-CAGCCCTGCTCACAGGCAAG-3', probe 5'-CTGGCTGTGATCCTGAGCT-3'; and β-actin, forward 5'-GAAGATCAAGATCATTGCTCCT-3', reverse 5'-TACTCCTGCTTGCTGATCCA CA-3', probe 5'-TCACTGTCCACCTTCCAGCAG-3'. The The PCR reaction mixture consisted of 3.0 mL 10 × buffer, 0.3 μL Taq enzyme (5 U/mL), 5.0 μL cDNA, 3 μL $MgCl_2$ (25 mM), 0.36 μL dNTPs, 1 μL of each primer (10 μM), 1 μL of Taqman probe (10 μM) and 15.34 μL of ddH_2O. The amplifying conditions 94 °C for 20 s, annealing for 20 s at a gene-specific temperature (REDD1, mTOR, GLUT1 and IκBα were 56 °C; TLR4 was 50 °C; NF-κB and β-actin were 54 °C) and 60 °C for 30 s. All samples were run three separate times and each time in triplicate in 96-well optical PCR plates (Applied Biosystems, California, USA). The expression levels of mRNA were standardized against those of β-actin.

Western blotting

Liver tissues were washed twice with phosphate buffer solution and lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1% Triton X-100). Protein concentrations were detected by BCA assay and enzyme equipment. The lysate (with 30 μg protein) was then resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis or 10% bistris acrylamide gradient gel (Invitrogen Life Technologies, Shanghai, China), and the membrane was incubated in a blocking solution containing 0.1% TBS-Tween 20 and 5% milk at room temperature for 1 h. Next, the protein was transferred onto a nitrocellulose membrane. The membrane was immunoblotted separately with monoclonal or polyclonal antibodies separately: anti-REDD1 (1:300; Abcam, Cambridge, UK, Cat. No. ab106356), anti-mTOR (1:500; Proteintech Group, IL, USA, Cat. No. 66888-1), anti-GLUT1 (1:1000; Proteintech Group, Cat. No. 21829-1), anti-p65 (1:1 000; Proteintech Group, Cat. No. 66535-1), anti-TLR4 (1:600; Proteintech Group, Cat. No. 19811-1), anti-IκBα (1:1000; Abcam, Cat. No. ab32518), and β-actin (1:5 000; Proteintech Group, Cat. No. 60008-1-Ig). After being washed 3 times with tris buffered saline with tween (TBS-T), the membrane was incubated with horse radish peroxidase (HRP)-conjugated secondary antibody (1:10 000; Gene C Ltd., Beijing, China) at room temperature for 2 h. All protein expression was quantified using the Molecular Analyst Software (Bio-Rad Laboratories, CA, USA). The intensity ratios of β-actin were obtained to quantify the relative protein levels.

Statistical analysis

Normally distributed continuous variable was presented as the mean ± standard deviation (*SD*), and the intergroup comparisons were performed with a one-way analysis of variance (ANOVA). Median and interquartile range were calculated for non-normal distributed continuous data and the comparisons among groups were analyzed with the nonparametric Kruskal-Wallis test. All data analysis was performed with SPSS 20.0 for Windows (SPSS Inc., IL, USA). A two-tailed $P < 0.05$ was considered statistically significant.

Results

Maternal characteristics

The pre-pregnancy rat body weight, number of fetal rats, fetal body weight, and placental weight were comparable among the four groups in the study ($P = 0.670$; $P = 0.160$; $P = 0.200$; $P = 0.150$) (Table 1).

Maternal serum biochemical parameters

The EE-sham and EE-IR groups showed significantly higher levels of maternal serum ALT (78.00 ± 20.05 *vs.* 43.25 ± 11.06, $P = 0.032$; 80.50 ± 16.74 *vs.* 45.25 ± 11.00, $P = 0.041$), AST (154.50 ± 36.35 *vs.* 80.75 ± 15.31, $P = 0.029$; 173.25 ± 21.28 *vs.* 84.50 ± 7.42, $P = 0.035$), and TBA (51.88 ± 9.75 *vs.* 17.88 ± 7.41, $P = 0.026$; 58.27 ± 15.67 *vs.* 15.58 ± 5.00, $P = 0.045$) compared with those in the control-sham and control-IR groups, respectively. No differences were found between control-sham and control-IR groups, as well as EE-sham and EE-IR groups in maternal serum levels of ALT (43.25 ± 11.06 *vs.* 45.25 ± 11.00, $P = 0.462$; 78.00 ± 20.05 *vs.* 80.50 ± 16.74, $P = 0.251$), AST (80.75 ± 15.31 *vs.* 84.50 ± 7.42, $P = 0.854$; 154.50 ± 36.35 *vs.* 173.25 ± 21.28, $P = 0.623$), and TBA (17.88 ± 7.41 *vs.* 15.58 ± 5.00, $P = 0.523$; 51.88 ± 9.75 *vs.* 58.27 ± 15.67, $P = 0.198$) (Fig. 1).

Protein expression in liver tissues

REDD1 expression in the liver tissues of the control-IR and EE-IR groups was significantly higher than that in the control-sham group (3.24 ± 0.98 *vs.* 1.06 ± 0.24, $P = 0.025$;

Table 1

Maternal characteristics of pregnant rats in the four groups.

Items	Groups				F	P
	Control-sham ($n = 6$)	EE-sham ($n = 6$)	Control-IR ($n = 6$)	EE-IR ($n = 6$)		
Pre-pregnancy body weight (g)	273.43 ± 9.78	272.50 ± 8.66	278.75 ± 9.28	270.30 ± 9.12	0.53	0.670
Number of fetal rats	11.00 ± 2.94	10.25 ± 2.50	13.75 ± 0.50	12.00 ± 1.63	2.06	0.160
Fetal body weight (g)	1.30 ± 0.16	1.24 ± 0.17	1.33 ± 0.19	1.27 ± 0.25	5.43	0.200
Placental weight (g)	0.51 ± 0.08	0.43 ± 0.09	0.52 ± 0.06	0.49 ± 0.03	4.95	0.150

Data are presented as means ± standard deviation.

EE: Estrogen; IR: Ischemia-reperfusion.

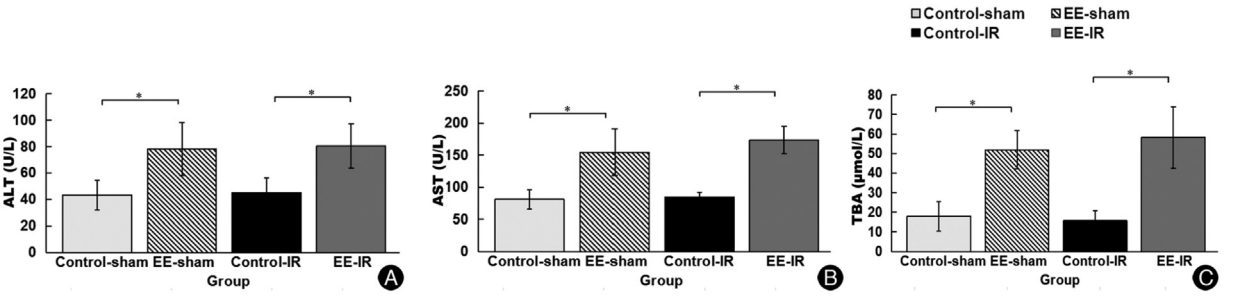

Figure 1. Maternal serum biochemical parameters of pregnant rats in the four groups. Maternal serum ALT (panel A), AST (panel B), and TBA (panel C) levels at day 19 of gestation in the control-sham, control-IR, EE-sham, and EE-IR groups ($n=6$). Data are presented as mean and standard deviation. $^{*}P<0.05$. ALT: Alanine transaminase; AST: Aspartate transaminase; EE: Estrogen; IR: Ischemia-reperfusion; TBA: Total bile acid.

4.46±0.65 *vs.* 1.06±0.24, $P=0.004$). Hepatic REDD1 levels in the EE-IR group were also markedly upregulated compared with those in the EE-sham group (4.46±0.65 *vs.* 2.05±0.47, $P=0.009$). No statistically significant differences were found between the control-sham and EE-sham groups in the hepatic REDD1 expression (1.06±0.24 *vs.* 2.05±0.47, $P=0.380$). The liver expression of mTOR in the control-IR and EE-IR groups was noticeably decreased compared with that in the control-sham (0.50±0.07 *vs.* 1.01±0.03, $P=0.001$; 0.42±0.09 *vs.* 1.01±0.03, $P=0.006$) and EE-sham groups (0.50±0.07 *vs.* 0.90±0.14, $P=0.002$; 0.42±0.09 *vs.* 0.90±0.14, $P=0.008$), respectively. No statistically significant changes of hepatic mTOR levels were observed between the control-sham and EE-sham groups (1.01±0.03 *vs.* 0.90±0.14, $P=0.077$), or the control-IR and EE-IR groups (0.50±0.07 *vs.* 0.42±0.09, $P=0.483$), respectively. Hepatic GLUT1, TLR4, and p65 expression in the control-IR group were obviously increased compared with those in the control-sham group (2.37±0.82 *vs.* 1.09±0.10, $P=0.039$; 2.12±0.29 *vs.* 1.20±0.28, $P=0.010$; 2.09±0.85 *vs.* 1.04±0.06, $P=0.023$). However, in the EE-IR group, the GLUT1, TLR4, and p65 expression in the liver tissues demonstrated no statistically significant differences compared with those in the EE-sham group (2.02±0.45 *vs.* 1.79±0.39, $P=0.240$; 2.10±0.74 *vs.* 1.60±0.36, $P=0.129$; 2.41±0.83 *vs.* 1.65±0.46, $P=0.145$). Subjecting EE-treated rats to placental IR did not further alter liver levels of GLUT1, TLR4, and p65 (2.02±0.45 *vs.* 2.37±0.82, $P=0.453$; 2.10±0.74 *vs.* 2.12±0.29, $P=0.601$; 2.41±0.83 *vs.* 2.09±0.85, $P=0.116$). IκBα expression in the liver tissues of the EE-sham group was significantly lower than that in the control-sham group (0.72±0.20 *vs.* 1.01±0.07, $P=0.008$). Meanwhile, hepatic IκBα levels in the control-IR group and EE-IR group were markedly downregulated compared with those in the control-sham (0.61±0.08 *vs.* 1.01±0.07, $P=0.014$; 0.43±0.09 *vs.* 1.01±0.07, $P=0.016$) and EE-sham (0.61±0.08 *vs.* 0.72±0.20, $P=0.013$; 0.43±0.09 *vs.* 0.72±0.20, $P=0.004$) groups, respectively (Fig. 2). Giving rats either EE alone or placental IR alone decreased hepatic expression of IκBα compared with that in the control rats.

mRNA expression in liver tissues

No obvious changes were observed among control-IR, control-sham, EE-IR, and EE-sham groups for mRNA expression of REDD1 (3.48(0.97) *vs.* 4.00(15.94) *vs.* 1.00(0.70) *vs.* 8.93(31.92), $P=0.296$), mTOR (1.23(0.41) *vs.* 1.00(0.78) *vs.* 1.00(0.71) *vs.* 1.12(1.79), $P=0.679$), GLUT1 (4.59(0.59) *vs.* 3.48(10.70) *vs.* 5.12(3.51) *vs.* 8.00(2.20), $P=0.140$), TLR4 (1.00(1.68) *vs.* 1.96(3.23) *vs.* 1.00(0.54) *vs.* 4.83(1.66), $P=0.106$), p65 (3.25(0.42) *vs.* 1.74(0.38) *vs.* 1.74(1.83) *vs.* 1.62(0.31), $P=0.226$), and IκBα (1.15(0.48) *vs.* 0.81(0.06) *vs.* 1.24(0.81) *vs.* 1.47(0.90), $P=0.111$) in livers tissues (Fig. 3).

Discussion

ICP is a pregnancy-specific liver disorder. It had been commonly accepted that the etiopathogenesis of ICP predominately involves the liver and placenta.[1,3,5] During pregnancy, the liver and placenta are closely related to each other for bile acid transportation, immune activities, and metabolism. Wang *et al.*[23] analyzed the correlations of maternal serum cholylglycine with umbilical artery systolic-to-diastolic ratio in ICP. They concluded that the maternal serum cholylglycine levels were positively correlated with umbilical artery systolic-to-diastolic ratio. This clinical correlation also indicating the close relationship between liver and placenta.

Meanwhile, existing research studies showed that bile acid transporters transferred bile salt bidirectionally between the mother and the fetus.[24,25] Studies have demonstrated that the expression of bile acid transporters in placentas from ICP were obviously down-regulated compared with those in normal pregnancy, implying that there is a possible change of bile acid component in maternal serum and the subsequent burden on the maternal liver.[26] Furthermore, there is evidence that the dysfunction of hepatic bile salt transporters resulting from genetic mutations also contribute to the pathogenesis of ICP.[8,27] However, the expression status of immunological and metabolism-related factors in liver tissues of ICP remains unclear.

In this study we established an EE-induced ICP rat model and its placental-fetal IR stress model according to the literature.[21,22] In order to avoid the possible bias introduced by solvent and the stimulation of injection, we used the rats received propylene glycol injection as control group. Because ICP is characterized by increased TBA, ALT, and AST levels, and most of the studies detected ALT and AST rather than gamma-glutamyl transpeptidase and alkaline phosphatase to evaluate the condition of ICP.[2] In this study, we detected ALT and AST

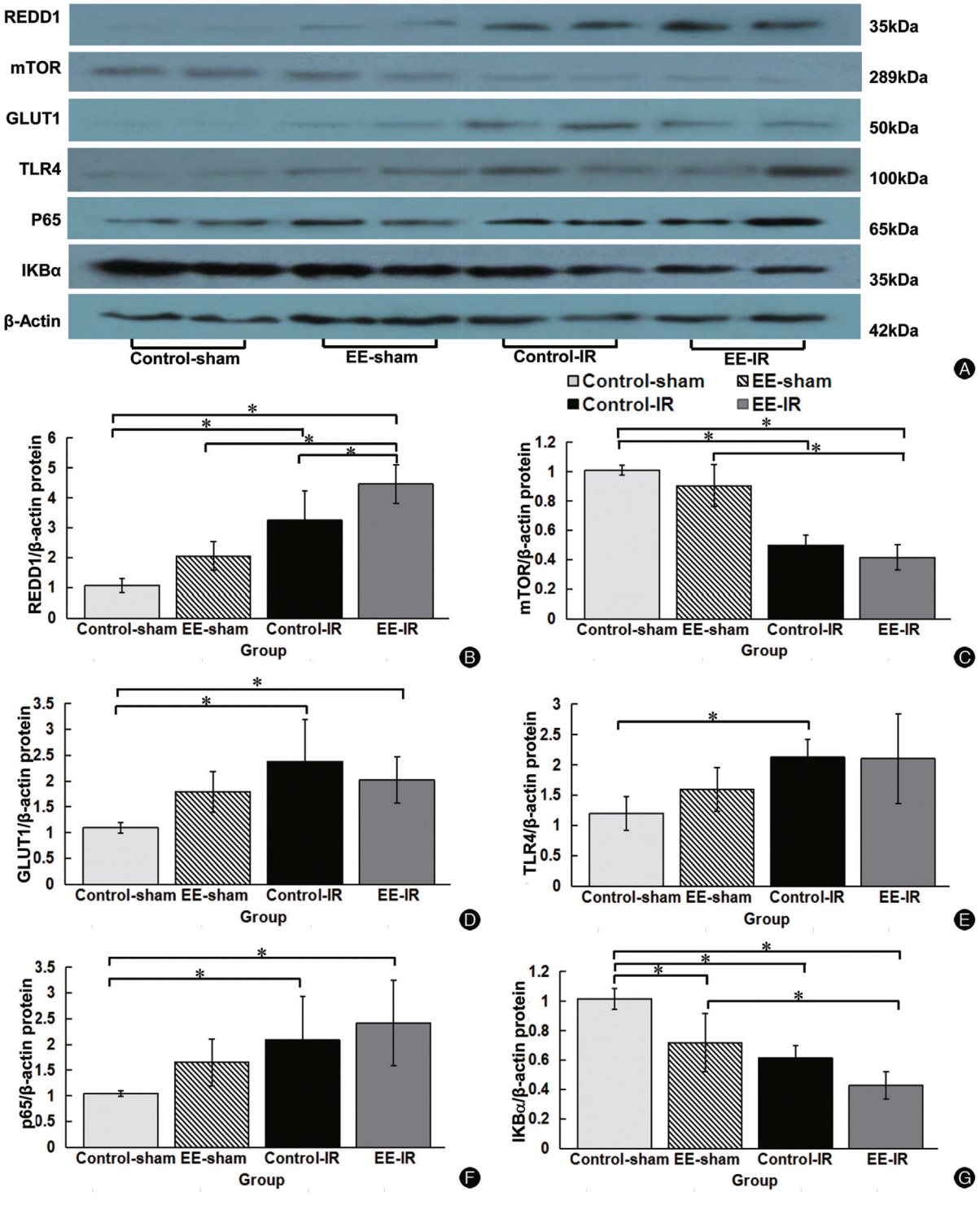

Figure 2. REDD1, mTOR, GLUT1, TLR4, p65, and IκBα protein expression in liver tissues from pregnant rats. A Electrophoretic bands are shown. B– G Relative protein expression levels of REDD1 (Panel B), mTOR (panel C), GLUT1 (panel D), TLR4 (panel E), p65 (panel F), and IκBα (Panel G) were normalized to beta-actin protein ($n = 6$). *$P < 0.05$. EE: Estrogen; GLUT1: Glucose transporter type-1; IR: Ischemia-reperfusion; mTOR: Mammalian target of rapamycin; REDD1: Regulator DNA damage response-1; TLR4: Toll-like receptor-4; IκBα: Inhibitor κBα. Data are presented as mean and standard deviation.

to make sure the serum biochemistry of animal model is in accordance with ICP. The maternal serum ALT, AST, and TBA levels in the EE groups were significantly higher than those in the control groups, revealing that our animal models were successfully established. Meanwhile, in our previous experiment, the placental levels of HIF-1α, marker of uterine-placental-fetal IR, were significantly elevated in pregnant rats of IR group compared with those in sham groups.[20] The liver levels of IκBα in EE-induced ICP rats were obviously decrease compared with the level in control pregnant rats. IκBα is the inhibitor of NF-κB and the compromised hepatic IκBα expression led to enhanced

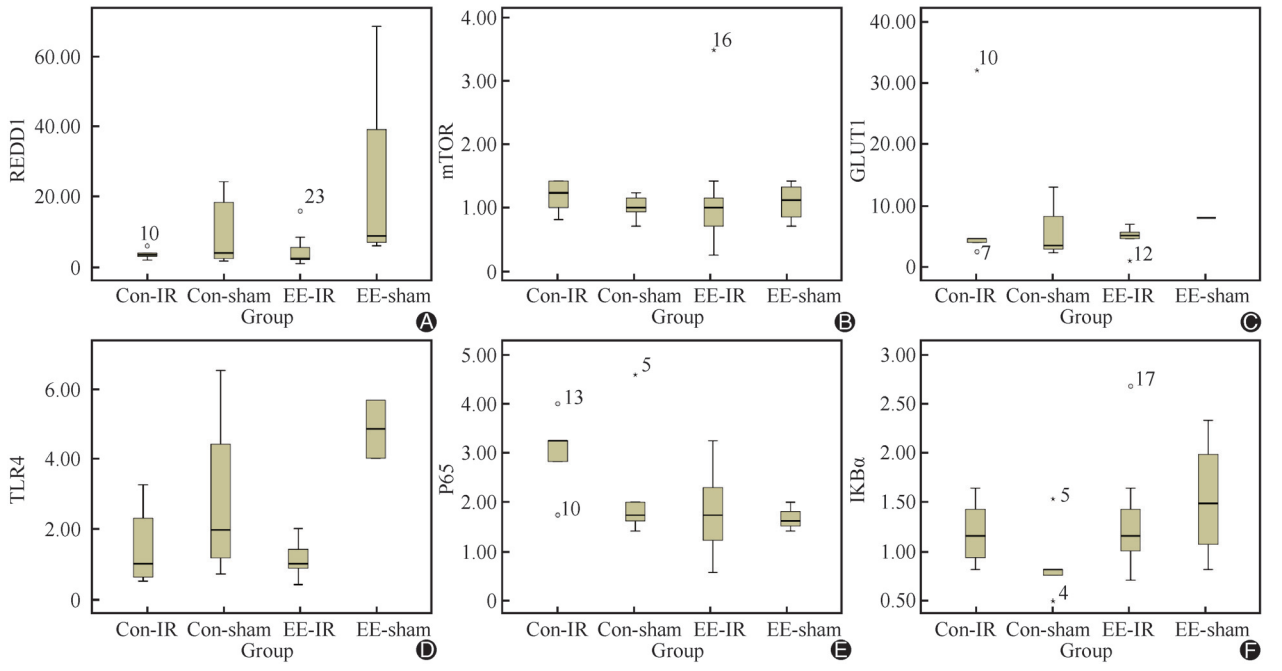

Figure 3. Hepatic expression of REDD1, mTOR, GLUT1, TLR4, p65, and IκBα mRNA in pregnant rats. Quantitative mRNA expression of REDD1/beta-actin (panel A), mTOR/beta-actin (panel B), GLUT1/beta-actin (panel C), TLR4/beta-actin (panel D), p65/beta-actin (panel E), and IκBα/beta-actin (panel F) ($n=6$). Data are presented as median and interquartile range (IQR). °outliers, value between one and a half box lengths of IQR and three box lengths of IQR; * extremes, value out of three box lengths of IQR. EE: Estrogen; GLUT1: Glucose transporter type-1; IR: Ischemia-reperfusion; mTOR: Mammalian target of rapamycin; REDD1: Regulator DNA damage response-1; TLR4: Toll-like receptor-4; IκBα: Inhibitor κBα.

activity of NF-κB.[11] Although p65 and TLR4 expression in the liver tissues of EE-induced ICP rats showed a slight increase compared with control rats, no statistically significant differences were found between EE rats and control rats. These results imply the distinct role of NF-κB in the placenta and liver from ICP, as other researches have shown that the placental NF-κB expression was increased in ICP patients and taurocholate acid treated trophoblast cells, and that bile acids could activate the NF-κB in trophoblasts.[6,28] Furthermore, no significant changes were observed in the liver expression of REDD1, mTOR, and GLUT1 between the EE-induced ICP rats and the control rats. By contrast, in our previous study, the REDD1, mTOR, and GLUT1 levels demonstrated obvious alteration in placentas from EE-induced cholestasis pregnant rats compared with the control rats.[28]

There is evidence that mTOR signaling pathway and endoplasmic reticulum stress were activated in the ICP placentas.[29] In studies reported by Chao *et al.*,[29] they used HTR-8/SVneo cell line and treated them with different dose of taurocholic acid and ursodeoxycholic acid. Results indicated that bile acid could activate mTOR signaling and lead to the decrease of cell viability. Nabih *et al.*[30] revealed significant increase of hepatic TLR4 and NF-κB expression in bile duct legation mice, and indicated that TLR4 participants in the cholestatic liver injury.

We further investigated the influence of placental IR stress on hepatic expression of REDD1-mTOR-GLUT1 and TLR4/NF-κB/IκBα in EE-induced ICP rats. Liver levels of REDD1-mTOR-GLUT1 and TLR4/NF-κB/IκBα in the control-IR rats were altered compared with those in the control rats, demonstrating the possible impact of placental IR stress on immune activities of the livers. Moreover, in the EE-induced ICP rats, hepatic REDD1, mTOR, and IκBα expression responded efficiently in placental IR models, whereas liver expression of GLUT1, TLR4, and NF-κB were impaired after being subjected to placental IR. Conversely, placental REDD1 and mTOR were proved to be compromised in EE-induced ICP rats with IR stress.[27] This notable difference of REDD1-mTOR-GLUT1 impairment implied the distinct regulatory mechanism between livers and placentas from EE rats with placental IR stress.

This study has limitations. First, we did not use a control group without any treatment or reagents. Second, the activation status of mTOR and p65 are not detected in this study. Further researches are needed in investigating the underlying regulatory mechanism between REDD1-mTOR-GLUT1 and TLR4/NF-κB/IκBα signal pathways.

This study investigated the expression status of immunologic and metabolism-related factors in liver tissues of EE-induced ICP. The REDD1-mTOR-GLUT1 signal pathway and TLR4/NF-κB/IκBα signal pathway demonstrated obvious change of protein levels after subjecting placental IR stress on EE-induced ICP rats. Moreover, the hepatic GLUT1, TLR4, and p65 regulation in IR stress were impaired in EE-induced ICP rats.

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Author Contributions

Fan Zhou and Yayi Hu conceived the idea and designed the experiments. Fan Zhou, Huafang Chen, Dan Shan, Yuxia Wu, and Qian Chen contributed to the animals experiments, data analysis, and manuscript drafting. Yayi Hu helped with the editing of the article and final approval of the version to be published.

Conflicts of Interest

None.

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