Reproductive Toxicology 65 (2016) 179–186

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Dynamic expression of corticotropin-releasing hormone and urocortin in estrogen induced-cholestasis pregnant rat

Fan Zhou[a,b], Bingxin Gao[a,b], Chunyan Deng[a,b], Guiqiong Huang[a,b], Ting Xu[a,b], Xiaodong Wang[a,b,*]

[a] Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University, Chengdu 610041, China
[b] Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Chengdu 610041, China

ARTICLE INFO

Article history:
Received 21 October 2015
Received in revised form 25 July 2016
Accepted 29 July 2016
Available online 1 August 2016

Keywords:
Placenta
Corticotropin-releasing hormone
Urocortin
Rat
Cholestasis
Regulation
Pathogenesis

ABSTRACT

Intrahepatic cholestasis of pregnancy(ICP) is complicated by acute placental-fetal hypoxia. Corticotropin-releasing hormone(CRH) and urocortin(UCN) are vasodilatory regulators of blood flow in the placenta. An ethinylestradiol(EE)-induced cholestasis rat model was reproduced and serum/placental CRH/UCN were detected during 14–21 days of gestation(DG). Maternal serum or placental CRH/UCN levels in the control rats were relatively consistent during 14–21DG. Serum CRH was reduced in the EE-treated rats compared with the control rats at 21DG. Regarding serum UCN, we observed a decrease at 17DG as well as an increase at 21DG in the EE-treated rats compared with the controls. Moreover, we observed a noticeable reduction of placental CRH/UCN expression at 17 or 19DG in the EE-treated rats compared with the control rats. The serum bile acids levels exhibited an inverse correlation with placental CRH/UCN expression. EE-induced cholestasis rats might serve as a good model to further investigate the pathological mechanism underlying CRH/UCN dysregulation in ICP.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disease in the second or third trimester of pregnancy. The incidence of ICP varies between 0.2% and 2% (in most areas of Europe, America and Asia), is dependent on ethnicity and geographic location and is reported to be 5% in the Yangtze River area of China [1,2]. ICP is characterized by maternal pruritus and abnormally increased maternal serum bile acids and liver transaminases levels, which rapidly return to normal after delivery. The etiology and pathogenesis of ICP has not been fully revealed. Several factors, such as genetics, female sex hormones and immunological and environmental factors, are thought to contribute to the onset of ICP. ICP can affect the fetus and lead to preterm birth (spontaneous or iatrogenic), meconium-stained amniotic fluid and intrauterine fetal death. The prenatal mortality of ICP is as high as 2.25% [3].

There is evidence that the sudden intrauterine fetal death in ICP was associated with acute intrauterine fetal hypoxia, which might be related to blood flow dysregulation of the utero-placental-fetal unit [3,4]. In normal conditions, the utero-placental-fetal unit has more than two times of the blood oxygen volume reserve compared with those in hypoxia-stressed conditions. The intervillous space, the lobular vascular volume and their blood update speed in the utero-placental-fetal unit are key compensatory elements for hypoxia. However, ICP patients were found to be complicated with acute utero-placental-fetal unit insufficiency—the placental intervillous space was decreased by 30%, and the lobular vascular volume was decreased by 29% [5–8]. Meanwhile, the mechanism of blood flow regulation in the human placenta is complex and is characterized by high blood flow volume, low vascular resistance and lack of neural regulation [9,10]. The local production of circulation-derived vasoactive substances, including prostaglandins, endothelium-derived relaxing factor, vasoactive peptides and the corticotropin-releasing hormone (CRH) family, especially CRH and urocortin (UCN), is important in blood flow regulation and hypoxia compensatory mechanisms in the utero-placental-fetal unit. These mechanisms increase the ability to cope with acute fetal hypoxia in late pregnancy and intrapartum fetal asphyxia [9–11].

Abbreviations: ICP, intrahepatic cholestasis of pregnancy; CRH, cotricotrophin releasing hormone; UCN, urocortin; EE, ethinylestradiol; CRH-R, cotricotrophin releasing hormone receptor; IUFD, intrauterine fetal death; ALT, alanine transaminase; AST, aspartate transaminase.

* Corresponding author at: Department of Obstetrics and Gynecology, Sichuan University, Renmin South Road, Chengdu, Sichuan, China.
E-mail address: wangxd_scu@sina.com (X. Wang).

http://dx.doi.org/10.1016/j.reprotox.2016.07.019
0890-6238/

In our previous study, the maternal serum and placental CRH/UCN expression were down-regulated in ICP patients [12,13]. However, the placental tissues of ICP patients can only be obtained after delivery, and the patients had always received multiple treatments for ICP. Subcutaneous administration of high doses of ethinylestradiol (EE) in rats induces a predictable and reversible cholestasis [14], and the mechanism of cholestasis is associated with alterations in the fluidity and lipid composition of the liver plasma membrane, driving force for the bile flow, Na^+-K^+-ATPase activity and bile salt transports [15–20]. EE-induced cholestasis pregnant rat serves as an excellent model in various studies relevant to the etiology and pathogenesis of ICP [21,22]. There is also evidence about the expression of CRH in decidualized endometrium and maternal decidua of Sprague-Dawley rats, as well as the involvement of locally produced CRH in mouse embryo implantation and pregnancy maintenance [23–25]. Both of them are in agreement with the hypotheses postulated in humans [11]. Thus, to further evaluate the placental CRH/CRH-R1 and UCN/CRH-R2 expression dynamically and explore their roles in the pathological mechanism of sudden intrauterine fetal death in ICP, we reproduced an EE-induced cholestasis pregnant rat model according to the method reported by Crocenzi [21]. Maternal serum CRH/UCN levels and placental CRH/UCN/CRH-R1/CRH-R2 expression were detected in the EE-induced cholestasis pregnant rats and control rats during 14–21 days of gestation.

2. Materials and methods

2.1. Animals

Adult female Sprague-Dawley (SD) rats weighing 250–300 g were mated with adult male SD rats weighing 300–350 g and were checked for vaginal plugs, which indicate pregnancy. The day a vaginal plug was observed was recorded as the first day of gestation. All of the rats were maintained on a standard water and diet *ad libitum* and were housed in a temperature (15–20 °C) and humidity (40%–70%) controlled room under a constant 12 h light/dark cycle. This study was approved by ethical committees at the West China Second University Hospital of Sichuan University. All rats received humane care according to the criteria prepared by the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Pregnant rats were randomly divided into two experimental groups at day 14 of gestation (n = 24). The EE group was subcutaneously administered EE daily (5 mg/kg body weight, propylene glycol as a solvent, the concentration of EE was 1 mg/ml, Sigma Chemical Co. Shanghai, China) for 5 days starting at day 14 of gestation [21]. The control group was subcutaneously administered propylene glycol (5 ml/kg body weight, Sigma Chemical Co. Shanghai, China) daily for 5 days starting at day 14 of gestation. Surgical procedures were performed at 14, 17 (the day after the third dose of EE or solvent was administered), 19 (the day after the last dose of EE or solvent was administered) and 21 days of gestation (3 days post administration). Animals were anesthetized with a single dose of chloral hydrate (2.5 mg/kg body weight, intraperitoneally). A middle abdominal incision was made rapidly. The placentas (exclude membranes) and blood were sampled within 5 min. At the end of each surgical procedure, the animals were sacrificed by exsanguination.

The blood samples were collected using an EDTA-containing tube and were centrifuged for 15 min at 1000 × *g* at 2–8 °C within 30 min. Alanine transaminase (ALT), aspartate transaminase (AST), total bile acids, total bilirubin and direct bilirubin levels were determined using a fully automatic biochemical analyzer (Siemens Electrical Apparatus Ltd., Germany). Rat maternal serum CRH or UCN levels were detected using a specific, commercially available ELISA kit (CUSABIO Life Science, China) according to the manufacturer's instructions. The pre-pregnancy bodyweight (recorded on the day before observation of vaginal plug), maternal bodyweight gain, number of fetal rats/litter, placenta weight (all placentas per litter), fetal rat's body weight (all foetuses per litter), meconium stained amniotic fluid and intrauterine fetal death were observed and recorded. The significance of meconium stained amniotic fluid in rats has not been fully elucidated. Studies from various types of animals indicated that the increase of utero defecation induced by fetal hypoxia, impaired clearance of meconium and decreased fetal swallow all contribute to meconium stained amniotic fluid [26].

2.2. Real-time PCR

Three placentas were randomly selected from each pregnant rats (except one rat in the control group only had three placentas) using simple random sampling. Total RNA was prepared from placental tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The purity and integrity of the RNA were evaluated by gel electrophoresis. RNA (200 μg), quantified by measuring the absorbance at optical density 260 (the ratio of optical density 260/optical density 280 around 1.9-2.0 means high purity of RNA), was reverse transcribed to cDNA using RNase Reverse Transcriptase (Life Technologies, Shanghai, China). All of the primers and probes used for real-time PCR were synthesized by Corelab Biotech (Chengdu, China). The primers were as follows: CRH, F: 5′-CAGCCGTTGAATTTCTTG-3′, R: 5′-GACTTCTGTTGAGGTTCC-3′; UCN, F: 5′-CAACGACGAGACGACC-3′, R: 5′-ACTTGCCCACCGAATC-3′; CRH-R1, F: 5′-GTGCCTGAGAAACATCAT-3′, R: 5′-ACCGAACATCCAGAAGAA-3′; CRH-R2, F: 5′-TGGTGACTTAGTGGACTA-3′, R: 5′-GAAGAGCATGTAGGTGAT-3′; and β-actin, F: 5′-CTGGAGAAGAGCTATGAG-3′, R: 5′-ATGATGGAATTGAATGTAGTT-3′. The probes were as follows: CRH, 5′-CAGCAACCTCAGCCGATTCT-3′; UCN, 5′-CCTCACCTTCCACCTGCTG-3′; CRH-R1, 5′-CACTGGAACCTCATCTCGGC-3′; CRH-R2, 5′-CATCATCCTCGTGCTCCTCATC-3′; and β-actin, 5′-ACGGTCAGGTCATCACTATCG-3′. PCR was performed in a reaction mixture consisting of FastStart Universal Probe Master (Roche, Shanghai, China) 10 μl, cDNA 5 μl, primer 2 μl, Taqman probe 1 μl and ddH_2O 2 μl. PCR program was 95 °C for 10 min; 95 °C for 15 s, 60 °C for 1 min (45 cycles) and 72 °C for 7 min followed by 4 °C (ABI 7900HT, Waltham, MA, USA). All samples were run in triplicate in 96-well optical PCR plates. For analysis, quantitative amounts of mRNA levels were standardized against the internal reference β-actin.

2.3. Western blotting

Placental tissues (100 mg) and 400 ml lysis buffer (with phenylmethane sulfonyl fluoride) were added to a homogenizing device and were homogenized for 30 min at 4 °C. The homogenate was centrifuged at 12,000 × *g* for 5 min at 4 °C, and the supernatant was collected. A bicinchoninic acid assay (Thermo Scientific™ Pierce™, Waltham, MA, USA) was used to determine the protein concentration. Then, samples were separated on an SDS-polyacrylamide gel, and the protein (50 μg) was electrophoretically transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, Massachusetts, USA). Next, the membranes were blocked in TBS-T (Tris-buffered saline containing 0.05% Tween 20) containing 5% non-fat dried milk for 2 h at room temperature and incubated with anti-CRH antibodies (Abcam, Shanghai, China) (1:10, 000), anti-UCN antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:800), anti-CRH-R1 antibodies (Proteintech, Rosemont, IL, USA) (1:350) or anti-CRH-R2 antibodies (Acris Antibodies,