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Prenatal ethanol exposure-induced adrenal developmental abnormality of male offspring rats and its possible intrauterine programming mechanisms

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ABSTRACT

Fetal adrenal developmental status is the major determinant of fetal tissue maturation and offspring growth. We have previously proposed that prenatal ethanol exposure (PEE) suppresses fetal adrenal corticosterone (CORT) synthesis. Here, we focused on PEE-induced adrenal developmental abnormalities of male offspring rats before and after birth, and aimed to explore its intrauterine programming mechanisms. A rat model of intrauterine growth retardation (IUGR) was established by PEE (4 g/kg·d). In PEE fetus, increased serum CORT concentration and decreased insulin-like growth factor 1 (IGF1) concentration, with lower bodyweight and structural abnormalities as well as a decreased Ki67 expression (proliferative marker), were observed in the male fetal adrenal cortex. Adrenal glucocorticoid (GC)-metabolic activation system was enhanced while gene expression of IGF1 signaling pathway with steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD) was decreased. Furthermore, in the male adult offspring of PEE, serum CORT level was decreased but IGF1 was increased with partial catch-up growth, and Ki67 expression demonstrated no obvious change. Adrenal GC-metabolic activation system was inhibited, while IGF1 signaling pathway and 3β-HSD was enhanced with the steroidogenic factor 1 (SF1), and StAR was down-regulated in the adult adrenal. Based on these findings, we propose a "two-programming" mechanism for PEE-induced adrenal developmental toxicity: "the first programming" is a lower functional programming of adrenal steroidogenesis, and "the second programming" is GC-metabolic activation system-related GC-IGF1 axis programming.

Introduction

Ethanol is one of the most widely consumed substances of abuse (Harris et al., 2008). Approximately 50% of women in reproductive age are binge drinkers in some communities, and nearly 7% of pregnant women consume alcoholic beverages (Muhajarine et al., 1997; Williams and Gloster, 1999). The prevalence rate of fetal alcohol syndrome is estimated to be between 2 and 7 cases in every 1000 births in the United States (May et al., 2009), and the rate of low birth weight is approximately 5.0%–7.4% (Mariscal et al., 2006; Patra et al., 2011). Prenatal ethanol exposure (PEE) may induce a variety of adverse fetal outcomes, including morphological abnormalities and fetal growth retardation (Lundsberg et al., 1997; Mullally et al., 2011; Murphy et al., 2013). Intrauterine growth retardation (IUGR) is defined as a birth weight and/or length below the 10th percentile for their gestational age and an abdominal circumference that is less than the 2.5th percentile with pathologic restriction of fetal growth (Eleftheriades et al., 2006). Environmental factors operating early in life can affect developing system, permanently altering the structure and function throughout life. The process with its persistent change in the functioning of parts of the genome is known as a "programming" effect (Neitzke et al., 2011). Our previous study showed that PEE resulted in fetal over-exposure to maternal glucocorticoids (GC) and inhibition of functional development of the fetal hypothalamic–pituitary–adrenal (HPA) axis (Liang et al., 2011). Furthermore, we found that the functional developmental suppression of the HPA axis in rats by PEE may persist after birth, even into adulthood. In these adults, the HPA axis showed low basal activity but hypersensitivity to chronic stress. The underlying mechanism involves a "HPA axis-related neuroendocrine metabolic programming alteration" (Xia et al., 2014). Interestingly, PEE-induced neuroendocrine metabolic programming alteration shows a partial trans-generational

Abbreviations: AKT1, protein kinase B; CEBPα, CCAAT/enhancer binding protein α; CEBPβ, CCAAT/enhancer binding protein β; CORT, corticosterone; CR, corticoid receptors; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GC, glucocorticoid (GC), GD, gestational day; GR, glucocorticoid receptor; HE, hematoxylin and eosin; HPA, hypothalamic–pituitary–adrenal; IUGR, intrauterine growth retardation; IGF1, insulin-like growth factor 1; IGF1R, IGF1 receptor; MS, metabolic syndrome; MR, mineralocorticoid receptor; P450scc, cytochrome P450 cholesterol side chain cleavage; PW, postnatal week; SF1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein; TEM, transmission electron microscopy; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2.

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effect with parental and gender differences (unpublished data). We also observed that PEE-induced IUGR offspring were much more susceptible to non-alcoholic fatty liver diseases (NAFLD) after birth fed with high-fat diet (Shen et al., 2014). Hence, we proposed an underlying mechanism of "HPA axis-associated neuroendocrine metabolic programming alteration" for PEE-induced enhanced susceptibility to metabolic syndrome and associated diseases (Liang et al., 2011; Shen et al., 2014; Xia et al., 2014).

The terminal effector of HPA axis, adrenal gland is responsible for GC synthesis and secretion that is of great significance in embryotic developments (Vieau et al., 2007). It has been demonstrated that adverse intrauterine environment impairs fetal adrenal development (Meaney et al., 2007). The long-term consequences of low birth weight on secretion of adrenal GC (cortisol in human and primates and corticosterone in rodents) may contribute to the increased risk of developing metabolic syndrome in later life, which suggests a central role for adrenocortical steroidogenesis (Ong, 2005; Marciniak et al., 2011). Therefore, adrenal steriodogenic function and circulating GC might be important for fetal maturation and growth (Mesiano and Jaffe, 1997). GC acting on corticoid receptors (CR) in target tissues is determined not only by circulating GC levels, but also by intracellular GC metabolism regulated by two 11β-hydroxysteroid dehydrogenases (11β-HSDs) isoforms: 11β-HSD1 and 11β-HSD2 (McNeil et al., 2007). CR comprises the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) that are both ligand-dependent nuclear transcriptional factors. CCAAT/enhancer-binding protein (C/EBP) family, in which isoforms of C/EBPα and C/EBPβ each act as essential collaborators in CR-mediated downstream gene expression (Esteves et al., 2012; Andrieu et al., 2014), is a critical group of bZIP transcriptional factors for the regulation of cell proliferation and development (Nerlov, 2007). As a core system of endocrine regulation, insulin-like growth factor 1 (IGF1) is a central factor in endocrine regulatory system, and always plays a role in IUGR caused by an unfavorable intrauterine environment and postnatal "catch-up growth" pattern. Numerous studies have confirmed that GC inhibit IGF1 expression in a variety of tissues and cells (Hyatt et al., 2007; Inder et al., 2010). The resulting question is whether PEE-induced adrenal developmental abnormality is associated with maternal high level of GC? Is there any intrauterine programming alteration? Does GC-metabolic activation system (11β-HSDs/CR/C/EBPs) get involved in? All of these issues are poorly defined.

Here, we established a rat IUGR model by PEE, and evaluated the adrenal developmental abnormalities of male offspring. Furthermore, we explored its intrauterine programming mechanisms by investigating the serum phenotypes, gene expression of adrenal GC-metabolic activation system, and IGF1 signaling pathway as well as steroidogenesis before and after birth. This work will be beneficial to better reveal the short- and long-term outcomes of ethanol developmental toxicity, and to clarify the susceptibility to metabolic syndrome in adult male offspring and its underlying mechanisms.

Materials and methods

Materials

Ethanol (analytical pure grade) was purchased from Zhen Xin Co., Ltd. (Shanghai, China). Isoflurane was obtained from Baxter Healthcare Co. (Deerfield, IL, USA). Rat corticosterone (CORT) ELISA kits were obtained from Assaypro LLC. (Saint Charles, Missouri, USA). Rat IGF1 ELISA kits were obtained from RD Systems, Inc. (Minneapolis, MN, USA). Trizol reagent was purchased from Invitrogen Co. (Carlsbad, CA, USA). Reverse transcription and quantitative PCR (Q-PCR) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Oligonucleotide primers for rat Q-PCR genes (PAGE purification) were custom synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All other reagents and chemicals were of analytical grade.

Animals and treatments

Specific pathogen free (SPF) Wistar rats (200–240 g) were purchased from the Experimental Center of Hubei Medical Scientific Academy (No. 2009-0004, Hubei, China). The animal experiments were performed in the Center for Animal Experiment of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine (Permit Number: 14016). All animal experiment procedures were approved by and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Animals were housed (room temperature: 18–22 °C; humidity: 40%–60%), after one week of acclimation, and two female rats were mated with one male rat for overnight. Upon confirmation of mating by the appearance of sperm in a vaginal smear, the day was taken as gestational day (GD) 0. Pregnant rats were transferred to individual cages and randomly divided into control and ethanol groups. The rats in the ethanol group were each dosed with ethanol at 4 g/kg·d by oral gavage from GD9 to GD20 to establish the stable rat IUGR model (Shen et al., 2014), while those of the control group were given the same volume (10 ml/kg) of distilled water. On GD20, the animals were placed in a separate quiet room for anesthesia using isoflurane. After the disappearance of the righting reflex, the animal was rapidly euthanized by cutting the left carotid artery with a sharp pair of scissors to collect maternal blood. Pregnant rats were selected with 10–14 live fetuses from each group ($n = 12$ for each group). Feto-placental units were quickly removed from the uterus, and the fetuses were weighed after being dried on filter papers. The IUGR rate was calculated according to the previously reported criteria for IUGR (Shen et al., 2014). Furthermore, male fetal serum samples from 3 littermates were pooled together and immediately frozen at −80 °C for analyses. Fetal adrenals were collected and three pairs of fetal adrenal glands were selected and routinely fixed by transcardial perfusion fixation (Yoshida and Ikuta, 1984) for histological and ultra-structural examination. Six pairs of fetal adrenals from two littermates were pooled for homogenization into one sample, and all pooled samples were immediately frozen and stored at −80 °C for gene expression analyses.

For the experiment with adult rats, pre-treatment of the animals was the same as the first portion. The pregnant rats were kept until normal delivery (GD21–GD22), and on postnatal day 1 (PD1) the numbers of pups were normalized to 10 pups per litter to assure adequate and standardized nutrition until weaning (postnatal week 4, PW4). After weaning, 10 male pups from ten different mothers were randomly selected from each group, and all pups were fed with lab chow (providing 22% of its energy content as protein, 63% as carbohydrate, and only 5% as fat) ad libitum. Bodyweights of the rat offspring were measured weekly, and the corresponding bodyweight gain rate was calculated as follows (Shen et al., 2014). At PW24, the offspring rats were anesthetized with isoflurane and decapitated. Serum was prepared and stored at −80 °C for analyses. Six adrenals were collected and routinely fixed for histological examination, and the rests immediately frozen and stored at −80 °C for gene expression analyses.

$$\text{Body weight gain rate } (\%) = \frac{\text{Body weight of PW x} - \text{Body weight of PW 1}}{\text{Body weight of PW 1}} \times 100$$

Analysis for blood samples

Serum corticosterone (CORT) and IGF1 concentrations were measured by ELISA assay kits following the manufacturer's protocol. The intra-assay and inter-assay coefficients of variation for CORT were 5.0% and 7.2%, respectively; cross-reactivity for the CORT ELISA was 2%