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The aryl hydrocarbon receptor is indispensable for dioxin-induced defects in sexually-dimorphic behaviors due to the reduction in fetal steroidogenesis of the pituitary-gonadal axis in rats

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ABSTRACT

Many forms of the toxic effects produced by dioxins and related chemicals take place following activation of the aryl hydrocarbon receptor (AHR). Our previous studies have demonstrated that treating pregnant rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a highly toxic dioxin, attenuates the pituitary expression of gonadotropins to reduce testicular steroidogenesis during the fetal stage, resulting in the impairment of sexually-dimorphic behaviors after the offspring reach maturity. To investigate the contribution of AHR to these disorders, we examined the effects of TCDD on AHR-knockout (AHR-KO) Wistar rats. When pregnant AHR-heterozygous rats were given an oral dose of 1 μg/kg TCDD at gestational day (GD) 15, TCDD reduced the expression of pituitary gonadotropins and testicular steroidogenic proteins in male wild-type fetuses at GD20 without affecting body weight, sex ratio and litter size. However, the same defect did not occur in AHR-KO fetuses. Further, fetal exposure to TCDD impaired the activity of masculine sexual behavior after reaching adulthood only in the wild-type offspring. Also, in female offspring, not only the fetal gonadotropins production but also sexual dimorphism, such as saccharin preference, after growing up were suppressed by TCDD only in the wild-type. Interestingly, in the absence of TCDD, deleting AHR reduced masculine sexual behavior, as well as fetal steroidogenesis of the pituitary-gonadal axis. These results provide novel evidence that 1) AHR is required for TCDD-produced defects in sexually-dimorphic behaviors of the offspring, and 2) AHR signaling plays a role in gonadotropin synthesis during the developmental stage to acquire sexual dimorphism after reaching adulthood.

1. Introduction

Dioxins are a class of environmental pollutants, and their harmful effects on health and development of humans, wildlife and their future generations continue to be of much concern. One of the reasons why dioxins are severe is their persistent nature due to the high lipophilicity and resistance to metabolic transformation. For example, the biological half-life of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic congener of dioxins, ranges from 2 to 4 weeks in most rodents [1], and that in adult humans is over 5 years [2]. It is known that TCDD produces a variety of toxic effects such as wasting syndrome, hepatotoxicity, tumorigenicity and immunotoxicity [3], which are triggered following activation of the aryl hydrocarbon receptor (AHR) [4,5]. AHR is a ligand-activated transcription factor which belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family. In the absence of ligands, AHR is present as a complex with heat shock protein 90, p23 and AHR-interacting protein in the cytoplasm [6]. Upon binding of the ligands including TCDD, AHR dissociated from the complex migrates to the nucleus, and dimerizes with another bHLH/PAS protein, AHR nuclear translocator. This heterodimer binds to the xenobiotic responsive element (XRE) in promoter regions of genes, including cytochrome P450 (CYP) 1A1 and other xenobiotic-metabolizing enzymes, to alter their expressions [7,8].

More importantly, maternal exposure to dioxin causes the impairment of reproduction and development in the offspring [9]. For example, *in utero* and lactational exposure to TCDD results in the offspring suffering from reduced body weight and length [10] and defects in sexually-dimorphic behaviors, such as sexual behavior and saccharin

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preference [11–13]. Several human studies have also suggested that maternal levels of dioxins and other AHR agonists are negatively linked to development, including body weight and sexual dimorphism and maturation of children [14–17]. In rats, although less than 0.1% of the TCDD dose exposed to pregnant dams is transferred to fetuses through the placental barrier [18,19], it causes developmental disorders in the offspring despite having little effects on dams exposed to TCDD. In addition, these effects continue even after the offspring reach maturity. For these reasons, the effects on later generations are extremely serious, and it is important to clarify the molecular mechanisms, including the contribution of AHR to these disorders.

A series of our previous studies have demonstrated that treating pregnant Wistar rats with TCDD at gestational day (GD) 15 reduces the pituitary production of gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] only during the fetal and neonatal period to attenuate the expression of steroidogenic proteins such as steroidogenic acute-regulatory protein (StAR) and CYP17 in their gonads [20,21]. Also, the reduced expression of LH is due to deacetylation of histones wrapped around its promoter region through the induction of histone deacetylase (HDAC) mRNAs in the fetal pituitary [21]. In addition, direct supplementation of equine chorionic gonadotropin, an LH-mimicking hormone, in fetuses restored the TCDD-induced impairment of sexual behaviors after reaching maturity as well as testicular steroidogenesis during the fetal stage [22,23]. These pieces of evidence strongly suggest that TCDD initially targets epigenetic regulation to attenuate the synthesis of pituitary LH during the fetal period, and this outcome leads to the imprinting of defects in sexual behaviors after reaching adulthood. Based on the above evidence, the present study was designed to examine the role of AHR in TCDD-induced defects in fetal steroidogenesis of the pituitary-gonadal axis and its outcomes on sexual maturation, using AHR-knockout (KO) rats generated by transcriptional activator-like effector nuclease (TALEN) technology [24].

2. Materials and methods

2.1. Materials

TCDD was purchased from AccuStandard, Inc. (New Haven, CT). Anti-rabbit StAR polyclonal antibody and anti-β-actin mouse monoclonal antibody were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX) and BioVision Inc. (Mountain View, CA), respectively. The other reagents were of the highest grade commercially available.

2.2. Animals and treatments

All experiments were approved by the Institutional Animal Care and Experiment Committee of Kyushu University. AHR-KO rats were produced using TALEN technology as described previously [24]. AHR heterozygous (AHR-Het) female and male rats were given sterilized tap water and standard chow (CE-2; CLEA Japan, Tokyo, Japan) *ad libitum*. They were kept in a room maintained at 22 ± 5 °C and 50 ± 15% relative humidity under a 24 h light/dark cycle (light period, 7:00 AM-7:00 PM). Female rats were paired overnight with male rats. The next morning, sperm in the vaginal smears was checked by microscopy (×400) to confirm pregnancy. The day when sperm was detected was designated as GD0 of pregnancy, and the pregnant rats were housed alone before the experiment was started. Considering the effect of the genetic background in dams, we obtained all offspring by crossing AHR-Het female rats onto AHR-Het males, and selected only the offspring of dams in which three different genotypes were included in one littermate (Fig. 1A). Pregnant AHR-Het rats at GD15 were given an oral dose of TCDD (1 μg/kg/2 ml corn oil), and the tissues and blood were collected from their wild-type, AHR-Het and AHR-KO fetuses at GD20. Our previous study confirmed that maternal exposure to TCDD reduces steroidogenesis of the fetal pituitary-gonadal axis in a dose-dependent manner, and its effective dose 50 is around 0.4 μg/kg [25]. Referring to the above evidence, this study used the maternal dose of 1 μg/kg TCDD which was enough to impair not only steroidogenesis during the fetal stage but also sexual behavior of the offspring after reaching maturity. Control dams were treated with corn oil alone. For the assessment of sexual behaviors and saccharin preference, fetuses were allowed to be born and develop before the experiments. Each genotype of litters was determined according to the method reported previously [24] (Fig. 1B), using the cDNA of the fetal pituitary or genomic DNA (gDNA) extracted from the ear of postnatal offspring.

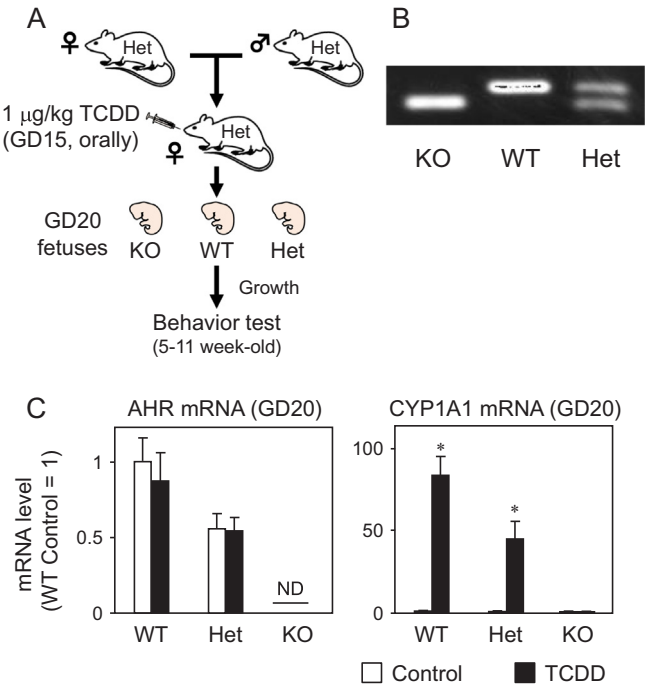

Fig. 1. Experimental design of this study and confirmation of AHR deletion. (A and B) Pregnant AHR-Het rats obtained by mating with AHR-Het males were treated with TCDD (1 μg/kg, orally at GD15). After determining the genotypes of their littermates (see panel B), three different genotypes included in a littermate were used for tissue collections and behavior tests. (C) The relative levels of AHR and CYP1A1 mRNAs in the fetal pituitary. Each bar represents the mean ± S.E.M. of 7–9 fetuses. The number of fetuses in each group is as follows. Control WT: N = 7, Control Het: N = 8, Control KO: N = 8, TCDD WT: N = 8, TCDD Het: N = 8 and TCDD KO: N = 9. These fetuses were removed from 5 Control and 7 TCDD-treated dams. Significantly different from the WT control: *, $p < 0.05$. Abbreviations: WT, wild-type; Het, heterozygote; KO, AHR-knockout; ND, not detectable.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

The fetal pituitary and testis at GD20 were collected individually. The expression of mRNAs was quantified by real-time RT-PCR according to the method described previously [26]. Briefly, the total RNA was extracted from each tissue using RNeasy kits (QIAGEN GmbH, Hilden Germany). The cDNAs were synthesized by PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa-bio, Shiga, Japan). The RNA (150 ng) obtained was treated with gDNA Eraser to digest contaminating genomic DNA, and then reverse-transcribed to synthesize cDNAs. The cDNA of target mRNA was amplified with Fast SYBR Green Master Mix (Thermo-Fisher Scientific, Inc., Waltham, MA), using a StepOnePlus Real-time PCR system (Thermo-Fisher Scientific). The primer designs are shown in Table 1. The PCR conditions were as follows: 95 °C, 20 s-40 cycles (95 °C, 3 s-60 °C, 30 s). The amplification efficiency of each primer set (%, mean ± SEM of three analyses) was as follows: β-actin, 107 ± 6; LHβ, 96 ± 10; FSHβ, 93 ± 4; StAR, 100 ± 11; gonadotropin-releasing hormone receptor (GnRHR),