EFFECTS OF TCDD ON SEXUAL DIFFERENTIATION –INFLUENCE OF THE *IN UTERO* EXPOSURE ON FETUS BRAIN AROMATASE ACTIVITY IN RATS–

Masahiko Ikeda [1,3], Naomi Inukai [1], Tetsuo Mitsui [1], Hideko Sone [2,3], Junzo Yonemoto [2,3], Chiharu Tohyama [2,3] and Takako Tomita [1,3].

[1] University of Shizuoka, Graduate School of Health Sciences, 52-1, Yada, Shizuoka, Japan
[2] Chemical Exposure & Health Effects Research Team, National Institute for Environmental Studies, 16-2, Onogawa, Tsukuba, Ibaraki, Japan
[3] CREST, JST, 4-1-8, Honcho, Kawaguchi, Saitama, Japan

Introduction

In mammals the intrinsic pattern of the central nervous system development is assumed to be organized along lines that are appropriate for the female. In males, differentiation from this pattern occurs as a result of androgens produced by the testis. Aromatase cytochrome P450 present in specific brain area, hypothalamus-preoptic area, then converts the androgens to estrogens. Local estrogen formation in this area, induces the sexual differentiation of neural structure during central nervous system development, modulating endocrine functions and male sexual behavior. In the rat, brain aromatase activity appears after gestational day (GD) 16, increases abruptly after GD17 to peak levels on GD19, then declines to low but detectable levels on the postnatal day (PND) 2 [1]. In general, males display slight to moderately higher brain aromatase activity than females during early development. While androgens may regulate brain aromatase levels during postnatal development [2], the role of androgens during prenatal development is uncertain [1].

There is growing concern that environmental exposure of endocrine disruptors might be adversely affecting male reproductive development and functions in animals and humans. Endocrine disruptors such as diethylstilbestrol irreversibly evoke a male pattern in brain circuitry in fetuses when given during a critical period of gestation for sexual differentiation, whereas their effects on the adult male organism are reversible. Mably et al. [3-5] and Bjerke et al. [6] reported that the reproductive organs of male offspring are susceptible to a single exposure of TCDD on GD15. Male offsprings exhibited reduced weights of sex organs, daily sperm production and cauda eididymal sperm numbers; decreased responsiveness of the adult prostate to androgenic stimulation; partially feminized and demasculinized sexual behavior and feminized patterns of luteinizing hormone regulation. However, the underlying mechanisms by which TCDD exerts such effects are unknown.

In an attempt to clarify the effects of TCDD on sexual differentiation, we examined the influence of *in utero* TCDD exposure on brain aromatase activity in rat fetus (GD20) and pups (PND2).

Materials and Methods

Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (Andover, MA). [1β-^{3}H]-androsto-4-ene-3, 17-dione ([^{3}H]-androstenedione) was obtained from NEN (Boston, MA) and testosterone RIA kit from DPC (Los Angeles, LA).

Animals and treatments

Holtzman rats were obtained from Charles River Laboratories (Kingston, NY) and maintained in our laboratory under specific pathogen-free conditions. TCDD 200, 800 and 1600 ng/kg (TCDD 200, TCDD 800 and TCDD 1600 group) dissolved in corn oil was orally administered to pregnant rats on gestation day (GD) 15. Dams were anesthetized with ether and killed by exsanguination on GD16 and GD20. Fetal (GD20) blood was collected from the umbilical artery. Fetal brain was dissected and immediately frozen with dry ice powder. Blood from litters on PND2 was collected from the abdominal aorta and the brain was dissected and treated as described.

Assay for brain aromatase activity in brain

Frozen fetal brains were cut into 2 mm thick sections at the anterior and posterior 1 mm each from optic chiasma. The section containing the preoptic area was homogenized in 250 µl of 10 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl, 1 mM EDTA, 10 mM dithiothreitol and protease inhibitors, and centrifuged for 10 min at 1800 xg. Aromatase activity in the supernatant was assayed in terms of released ^3H_2O from ^{3}H-androstenedione as described by Lephart and Simpson [7] and Roselli and Resko [8]. Briefly, the enzyme solution (100 µl) was incubated with substrate solution (100 µl) containing 10 mM potassium phosphate buffer (pH 7.4), 100 mM KCl, 1 mM EDTA, 10 mM dithiothreitol, 2 mM NADPH, 20 mM glucose-6-phsphate, 2 U/ml glucose-6-phosphate dehydrogenase, 0.3 µM [^{3}H]-androstenedione for 1 hour at 37°C. The reaction was terminated by an addition of 1.5 ml of $CHCl_3$ and 1 ml of H_2O and mixed for 2 min. The mixture was then centrifuged for 20 min at 1500 xg. An aliquot of the supernatant (0.8 ml) was placed into tubes containing 0.8 ml of 0.5% dextran T-70 and 5% charcoal, vortexed for 1 min, and centrifuged for 15 min at 9000 xg. Radio activity in the supernatant (1.2 ml) was counted by liquid scintillation counter (LSC3100, Aloka, Tokyo, Japan).

Other methods

Protein concentration was measured as described by Lowry et al [9]. Testosterone in fetal serum was extracted with ether and measured by RIA.

Results and Discussion

In utero exposure of TCDD on GD15 did not influence fetal weight, litter numbers and sex ratio at both GD16 and GD20. While 3 and 13.4% fetuses of TCDD 800 and 1600 groups respectively, were found dead on GD20. At PND2, however, the exposure significantly and dose-dependently decreased litter numbers and pups weights. TCDD given on GD15 exerted more toxic effects on PND2 than GD20 and GD16. The half-life of TCDD in rats was approximately 3 weeks [10]. Maternally exposed TCDD on GD15 is transferred continuously to the

fetus through the placenta until birth, thus the fetus will be more affected by TCDD with development of age.

Aromatase activity in brain sections containing the preoptic area was significantly higher in male litters than in females on GD20 in the control group. The ratio of female / male was 0.7. TCDD exposure, however, increased significantly female / male ratios of brain aromatase activity on GD20 with the increasing doses ($P<0.05$ by ANOVA), and sex differences disappeared in TCDD 1600 group (Figure 1). Sex difference of brain aromatase activity in pups on PND2 also disappeared in TCDD 800 group. These results suggest that *in utero* TCDD exposure may induce demasculinization of male pups by inhibiting the aromatase activity in male brain hypothalamus-preoptic area. Plasma testosterone concentration is assumed to be crucial for the expression of brain aromatase during prenatal development. Serum testosterone concentration of fetuses on GD20 was significantly higher in male fetuses than in females in control group, while TCDD effect on testosterone concentration was not observed in either sex. Drenth et al. [11] reported that TCDD up to 0.1 μM did not exert any effect on the aromatase activity of human placental microsomes. Letcher et al. reported that TCDD was ineffective on aromatase activity and DNA content in JEG-3 cells at less than 0.1 μM concentration [12]. These results suggest that TCDD does not directly inhibit brain aromatase activity. It is not clear at present whether TCDD affects brain aromatase via arylhydrocarbon hydroxylase receptor.

In conclusion, *in utero* TCDD exposure on GD15 may induce demasculinization of male offsprings by inhibiting the brain aromatase activity during prenatal development.

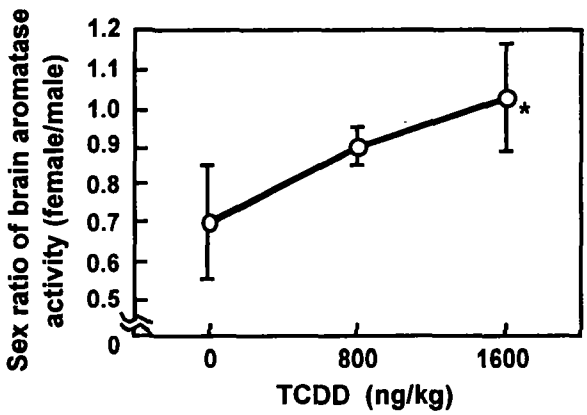

Figure 1 Changes in female to male ratio of fetal brain aromatase activity by *in utero* exposure of TCDD.

TCDD was orally given to dams on GD15, and dams were dissected on GD20. Coronal sections (2 mm thickness) containing preoptic area were dissected for aromatase assay.
Each point and vertical bar indicate mean±SD (n=3).
*Significance: $P<0.05$ versus none by ANOVA.

References

1. Lephart E.D., Simpson E.R. and Ojeda S.R. (1992) Mol Brain Res. 16, 187.
2. Roselli C.E. and Resko J.A. (1993) J Steroid Biochem. 44, 499.
3. Mably T.A., Moore R.W. and Peterson R.E. (1992) Toxicol Appl Pharmacol. 114, 97.
4. Mably T.A., Moore R.W., Goy R.W. and Peterson R.E. (1992) Toxicol Appl Pharmacol. 114, 108.
5. Mably T.A., Bjerke D.L., Moore R.W., Gendron-Fitzpatrick A. and Peterson R.E. (1992) Toxicol Appl Pharmacol. 114, 118.
6. Bjerke D.L., Sommer R.J., Moore R.W. and Peterson R.E. (1994) Toxicol Appl Pharmacol. 127, 250.
7. Lephart E.D. and Simpson E.R. (1991) Method Enzymol. 206, 477.
8. Roselli C. and Resko J.A. (1991) Neuroendocrine Research Methods. 2, 937.
9. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) J Biol Chem. 193, 265.
10. Abraham K., Krowke R. and Neubert D. (1988) Arch Toxicol. 62, 359.
11. Drenth H.-J., Bouwman C.A., Seinen W. and van den Berg M. (1998) Toxicol Appl Pharmacol. 148, 50.
12. Letcher R.J., van Holsteijn I., Drenth H.-J., Norstrom R.J., Bergman A., Safe S., Pieters R. and van den Berg M. (1999) Toxicol Appl Pharmacol. 160, 10.