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Letter

Distribution of ^{14}C-2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the brain and peripheral tissues of fetal rats and its comparison with adults

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ABSTRACT — Some forms of reproductive and developmental toxicity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) occur via initial damage to the pituitary synthesis of gonadotropins followed by the reduced expression of gonadal steroidogenic proteins. Defects in gonadotropin synthesis are highly specific to the periods from late fetal to early newborn stages. The reason for this specificity remains unknown. To address this issue, we compared the tissue distribution of ^{14}C-TCDD between fetal and adult rats. In adult male rats, the major portion of TCDD given orally (approximately 33-42% dose) accumulated in the liver during day 1 and 5 after treatment. Very little TCDD (approximately 0.01% of the dose) distributed into the brain. A similar picture was also observed in TCDD-treated pregnant rats. The amount of TCDD transferred from a dam to the fetuses was extremely low (around 0.02% of the maternal dose/fetus) after 1 day of treatment. Male and female fetuses showed the same pattern in the brain distribution of TCDD. The rate of TCDD distribution to fetal brain, which was calculated on the basis of body burden to a fetus, was 100 times or more than that in adults. However, the brain content of TCDD (ng/g tissue) was comparable in fetuses and their dams, and adult males exposed to TCDD. These results suggest that although TCDD easily translocates to fetal brain, this is not a major mechanism for a fetal age-specific reduction in gonadotropin synthesis.

Key words: Dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Distribution, Rat, Fetus

INTRODUCTION

Dioxins, represented by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are one of the typical and most common toxic environmental pollutants. They are believed to exert a variety of adverse effects in humans and wild animals via a mechanism involving the activation of the aryl hydrocarbon receptor (AhR) or oxidative stress (Stohs *et al.,* 1990; Alsharif *et al.,* 1994; Fernandez-Salguero *et al.,* 1995; Mimura *et al.,* 1997). It is well known that dioxins produce a number of adverse effects such as wasting syndrome, immunosuppression, carcinogenesis, and endocrine disruption (Poland and Knutson, 1982; Kogevinas, 2001). Among the toxic effects produced by dioxins are developmental and reproductive disorders. Some forms of these emerge after birth as delayed puberty (Gray *et al.,* 1995; Hurst *et al.,* 2000), a reduction in the sperm count (Gray *et al.,* 1995), accelerated senescence (Franczak *et al.,* 2006), and disorders of sexual behavior (Mably *et al.,* 1992; Bjerke *et al.,* 1994). Those injuries seem to be much more serious than non-reproductive toxicity, because they occur in pups following maternal exposure to TCDD at lower doses which cause no adverse effects on the dams. As for the mechanism of TCDD-produced reproductive and developmental toxicity, many of the research reports published so far have suggested an alteration in steroid hormones and their receptors by the AhR-signaling pathway. For example, suggested mechanisms include the following: 1) agonistic/antagonistic effects by activated AhR on estrogen receptor-dependent signaling (Chaffin *et al.,* 1996; Klinge *et al.,* 1999; Ohtake *et al.,* 2003), 2) a reduction in the expression of sex steroid

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receptors (Tian *et al.,* 1998; Theobald *et al.,* 2000, Ohsako *et al.,* 2001; Ohtake *et al.,* 2007), and 3) the induction of steroid-metabolizing enzymes (Spink *et al.,* 1990; Badawi *et al.,* 2000). However, it remains largely unknown how the above mechanisms or their combination contribute to reproductive and developmental toxicity. We have reported previously that treating pregnant rats with TCDD at gestational day (GD) 8 or 15 reduces the expression of steroidogenic proteins, including steroidogenic acute-regulatory protein (StAR) and cytochrome P450 (CYP) 17, in the fetal testis (Mutoh *et al.,* 2006; Taketoh *et al.,* 2007). This damage imprints defects in sexual behavior at adulthood (Takeda *et al.,* 2009). Our previous studies have also suggested that such a defect by TCDD is due to a reduction in the fetal expression of pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Mutoh *et al.,* 2006; Taketoh *et al.,* 2007). This is supported by evidence that the direct supplementation of exogenous LH to TCDD-exposed fetuses restores abnormal sexual behavior at adulthood (Takeda *et al.,* 2009). A notable finding is that the TCDD-induced downregulation of steroidogenic proteins and gonadotropins takes place only in the perinatal stages. The present study was designed to clarify the mechanism governing this specificity.

Based on its low molecular weight and high lipophilicity, TCDD is expected to distribute to and accumulate in the brain. However, in adult rodents, the distribution of TCDD into the brain is quite limited (Olson, 1986; Pohjanvirta *et al*., 1990; Weber *et al*., 1993). Those studies strongly suggest that the majority of ingested TCDD cannot enter the brain due to rejection by the blood-brain barrier (BBB). The maturation of the BBB appears to require the concomitant development of nerves, because capillary density correlates with the development of cortical function during early life (Argandoña and Lafuente, 1996). Although brain differentiation and development is still an active research field, vasculogenesis in the neural tube is thought to begin on embryonic day 10 to 11 in rats (Bar, 1980), and it continues until postnatal day (PND) 20 (Robertson *et al.,* 1985). A further period is needed for functional maturation. In general, the distribution of environmental toxicants into the fetal and infant brain is greater than that at adulthood (Andersen *et al.,* 2000), because of the immature BBB during developmental stages. Therefore, it is reasonable to hypothesize that the fetal age-specificity in TCDD-produced damage on pituitary gonadotropins is due to greater distribution of TCDD into the fetal brain having an immature BBB. To address this issue, we compared the tissue distribution of ^{14}C-TCDD between fetal and young-adult rats.

MATERIALS AND METHODS

Materials

^{14}C-TCDD (uniform labeling at a benzene ring, 4.3 TBq/mol) was purchased from BlyChem Ltd. (Billingham, U.K.). Its chemical structure was verified by electron-impact mass (MS) and proton nuclear magnetic resonance (NMR) spectroscopies: MS, 323.8 (M^+); and NMR, 7.27 ppm (singlet, 4H; solvent, $CDCl_3$). The radiochemical purity of ^{14}C-TCDD was confirmed to be over 98% by high-performance liquid chromatography: the retention time was 6.0 min under the following conditions: column, Phenomenex C18 (4.6 x 150 mm; Phenomenex Inc., Torrance, CA, USA); mobile phase, 0.1% trifluoroacetic acid-acetonitrile (7:3, v/v); flow rate, 1 ml/min; and oven temperature, 25°C. Soluent® 350, a tissue solubilizer, and Hionic-Fluor™, a scintillation cocktail, were purchased from Perkinelmer, INC. (Waltham, MA, USA). The other reagents were of the highest grade commercially available.

Animal treatments and tissue preparation

All experiments were pre-approved by the Institutional Animal Care and Experiment Committee of Kyushu University. Female Wistar rats (7 weeks-old) and male Wistar rats (10-20 weeks-old) were purchased from Kyudo Co. Ltd. (Kumamoto, Japan). All animals were bred on a standard chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and received sterilized water *ad libitum*, and they were kept in a room maintained at 22 ± 5˚C and 50 ± 15% relative humidity under a 24 hr light/dark cycle (light period, 7:00 AM-7:00 PM). Female rats were paired overnight with male rats in a ratio of 2:1 (female : male). Next morning, the presence of sperm in the vaginal smears was checked by microscopy (x 400) to confirm pregnancy. When sperm was detected, the day was designated as GD0 of pregnancy. In all experiments, pregnant rats at GD15 were given orally ^{14}C-TCDD (10 μg/kg body weight/2 ml corn oil x 1). The tissues, including blood, of the dams and their conceptuses were removed and weighted at GD16 or GD20. Control dams were treated with corn oil alone. Fetuses and placentas were removed from the conceptuses, and male and female fetuses were separated for further experiments. Among the fetal tissues, whole brain, liver and blood were taken for the measurement of radioactivity. Blood was kept for 2 hr at room temperature, and the serum was prepared by centrifugation at 2,500 r.p.m., 4˚C for 15 min. The data of TCDD distribution from all male and female fetuses in one dam were separately averaged to become one analyzing unit, and estimation was made using 3 dams. In a separate experiment, male and female fetuses were removed from 3 different dams for

assessing the body burden of TCDD. Prepared samples were stored at -30˚C until use.

In experiments using young-adult rats, male Wistar rats (7 weeks-old) were treated orally with ^{14}C-TCDD (10 μg/kg body weight/2 ml x 1), and the tissues and blood were collected 1 day or 5 days later. Control animals were treated with corn oil not containing ^{14}C-TCDD. Serum was prepared by the procedures described above.

Liquid scintillation counting

Tissues from young-adult and fetal rats were cut into 100~200 mg pieces and dissolved in 1 ml Soluent® 350 at 60˚C for 4 hr. A whole fetus was homogenized without buffer in a Waring blender, and a portion (1 g) of the homogenate was solubilized with 1 ml Soluent® 350 at 60˚C for 4 hr. Serum (1 ml) was treated at 60˚C for 2 hr in the presence of 1.0 ml isopropanol/Soluent® 350 (1:1) solution. All samples were then bleached with 0.5 ml 30% H_2O_2 at room temperature until oxygen generation ceased. To this solution 10 ml Hionic Fluor™ was added, and the radioactivity of the mixture was measured in a liquid scintillation counter, LSC-5100 (ALOKA Co., Ltd., Tokyo, Japan). Background radioactivity was determined using control tissues (or fetuses) from corn oil-treated rats (or dams), and this was subtracted from the radioactivity detected in animals exposed to ^{14}C-TCDD.

Statistical analysis

Statistical differences between two groups were calculated by Student's *t*-test. Statistical differences among multiple groups were evaluated by one-way analysis of variance with a post-hoc test (Fischer's Protected Least Significant Difference Method).

RESULTS

The tissue distribution of ^{14}C-TCDD in young-adult male rats and pregnant rats is shown in Table 1. In male rats at day 1 after treatment, the largest amount of TCDD was distributed to the liver (approximately 42% of the administered dose per whole organ). Although the accumulation of TCDD in the kidney and adrenal was relatively abundant, it was less than 0.5% of that in the liver. Other tissues including the brain contained smaller percentages, all of which were less than 0.1% of the dose. In agreement with this, the TCDD content per g tissue was far greater in the liver than in other tissues. At day 5 after treatment, the TCDD content decreased in all tissues compared with day 1, although the rate of reduction varied among tissues. However, the reduction rate in the brain during 4 days (from day 1 to day 5) (approximately 17%) was lower than that of other tissues (21-59%). In the case of pregnant rats at GD16 (day 1 after treatment), the largest percentage of TCDD was also detected in the liver (approximately 46% of the administered dose per whole tissue). Similarly to male adults, relatively large amounts of TCDD were detected in the kidney and adrenal, although the percentages were less than 0.2% of the dose. After 5 days, a reduction in the percentage distribution was observed in all tissues analyzed. Among them, the reduction rate during 4 days in the brain (approximately 14%) was relatively lower than that in other tissues (17-94%).

Table 2 shows the fetal distribution of TCDD following maternal exposure. As can be seen in the table, the translocation rate from a dam to a fetus (whole body) at GD16 was very low, and around 0.02% of the maternal dose in both female and male fetuses. Because the above value of body burden to fetuses was higher than the distribution to the placenta, this organ may not be an effective protector against TCDD. In sharp contrast to adult rats, TCDD easily distributed to male fetal brain, and the ratio to the body burden reached 15% of that in the liver. In this context, while the TCDD content on the basis of g tissue in fetal liver was much lower than that in adult liver, the brain content in male fetuses was not greatly different from that in adults (see also Table 1). Also, in female fetuses, a comparable TCDD distribution pattern to males was observed (3% in the brain and 20% in the liver on the basis of body burden). A marked difference between adults and fetuses was also seen in the TCDD re-distribution. That is, the hepatic and brain contents of TCDD remained unchanged or were increased rather than decreased in both male and female fetuses at GD20 compared with fetuses at GD16. In particular, the female content of TCDD in the whole body and the liver, and the male hepatic content were significantly increased during 5 days from GD16 to GD20.

DISCUSSION

Our previous studies have demonstrated that the fetal expression of both pituitary gonadotropins and steroidogenic proteins in the gonads was reduced by 1 μg TCDD/kg body weight (Mutoh *et al.,* 2006; Taketoh *et al.,* 2007; Takeda *et al.,* 2009). In the present study, we used a dose of 10 μg TCDD/kg because of the low specific radioactivity of the ^{14}C-TCDD available. Although this difference should be kept in mind, Hurst *et al*. (2000) have reported that the different ranges of TCDD dose have little effect on the profile of the tissue distribution of this compound. Therefore, it is expected that the pattern in the tissue dis-

Table 1. Tissue distribution of TCDD in adult rats

Tissue	Tissue content (% of dose/whole organ)			
	Male		Pregnant female	
	After 1 day	After 5 days	After 1 day	After 5 days
Brain	0.012 ± 0.007	0.010 ± 0.004	0.022 ± 0.003	0.019 ± 0.003
	(0.52 ± 0.03)[a]	(0.34 ± 0.01)*	(0.42 ± 0.02)	(0.35 ± 0.01)*
Thymus	0.018 ± 0.002	0.012 ± 0.008	0.026 ± 0.014	0.019 ± 0.004
	(0.90 ± 0.05)	(0.83 ± 0.02)	(1.03 ± 0.04)	(0.83 ± 0.03)*
Heart	0.019 ± 0.002	0.014 ± 0.007	0.020 ± 0.007	0.012 ± 0.007
	(0.57 ± 0.00)	(0.98 ± 0.02)*	(0.77 ± 0.02)	(0.67 ± 0.03)*
Lung	0.072 ± 0.022	0.039 ± 0.011	0.088 ± 0.027	0.042 ± 0.017
	(1.63 ± 0.02)	(1.13 ± 0.02)*	(1.88 ± 0.05)	(1.78 ± 0.10)
Stomach	0.043 ± 0.016	0.033 ± 0.007	0.065 ± 0.012	0.045 ± 0.007
	(0.90 ± 0.01)	(0.84 ± 0.02)*	(0.99 ± 0.02)	(0.80 ± 0.01)*
Liver	42.1 ± 5.5	33.1 ± 3.6*	46.4 ± 2.7	30.4 ± 3.6*
	(35.7 ± 0.4)	(26.7 ± 0.7)*	(36.3 ± 0.6)	(28.3 ± 0.4)*
Spleen	0.013 ± 0.003	0.009 ± 0.006	0.015 ± 0.011	0.010 ± 0.006
	(0.54 ± 0.00)	(0.50 ± 0.03)	(0.67 ± 0.03)	(0.67 ± 0.04)
Kidney	0.181 ± 0.022	0.074 ± 0.030*	0.152 ± 0.032	0.050 ± 0.030*
	(2.05 ± 0.01)	(1.09 ± 0.04)*	(1.92 ± 0.06)	(0.82 ± 0.07)*
Adrenal	0.152 ± 0.014	0.078 ± 0.007*	0.191 ± 0.022	0.011 ± 0.002*
	(4.36 ± 0.01)	(2.74 ± 0.16)*	(4.40 ± 0.08)	(1.60 ± 0.09)*
Gonad (Testis or ovary)	0.015 ± 0.004	0.007 ± 0.006	0.012 ± 0.013	0.010 ± 0.003
	(0.15 ± 0.01)	(0.12 ± 0.07)	(0.64 ± 0.05)	(0.44 ± 0.06)*
Serum	-	-	-	-
	(0.80 ± 0.01)	(0.52 ± 0.04)*	(0.98 ± 0.08)	(0.87 ± 0.05)

[a]The content expressed as ng TCDD/g tissue or ml serum is shown in parenthesis. Each value represents the mean ± S.D. of 3 animals. * Significantly different from the value obtained one day after TCDD treatment ($P < 0.05$).

tribution of TCDD observed in this study is close to that which would have taken place at 1 μg TCDD/kg.

This study revealed that the distribution of TCDD in fetuses one day after maternal exposure is extremely low (around 0.02% of the maternal dose). This rate of distribution was comparable between male and female fetuses, and it was increased or showed a trend to increase along with the exposure periods (Table 2). In spite of a lower body burden to fetuses, the rate of TCDD distribution to the brain on the basis of body burden was much greater in fetuses (eg., male fetuses, 2.9%) than in adults (e.g. male young-adults, approximately 0.01%). This evidence strongly suggests that the transfer of TCDD to the fetal brain is far easier than that to the adult brain. As mentioned in the introduction, the developmental and reproductive damage by dioxins seem to be produced, at least in part, by a reduction in gonadotropin synthesis which occurs in a fetal age-specific fashion. Thus, it is conceivable that a fetus-specific reduction in gonadotropins is due to the greater distribution of TCDD to the fetal brain, even

Table 2. Tissue distribution of TCDD in male and female fetuses in dams treated with TCDD at GD15

Tissue	Tissue content (% of maternal dose/whole organ x 1,000)			
	Male		Female	
	After 1 day (GD16)	After 5 days (GD20)	After 1 day (GD16)	After 5 days (GD20)
Whole body	24.3 ± 4.8	29.2 ± 3.8	19.8 ± 1.6	29.7 ± 0.3*
	(100)[a]	(100)	(100)	(100)*
	[0.591 ± 0.092][b]	[0.894 ± 0.122]*	[0.337 ± 0.033]	[0.913 ± 0.032]*
Brain	0.7 ± 0.1	1.0 ± 0.2	0.7 ± 0.4	0.9 ± 0.3
	(2.9 ± 0.4)	(3.4 ± 0.8)	(3.5 ± 2.0)	(3.0 ± 1.0)
	[0.248 ± 0.034]	[0.328 ± 0.054]	[0.193 ± 0.011]	[0.298 ± 0.032]*
Liver	4.6 ± 0.8	6.6 ± 0.9*	4.0 ± 0.4	6.0 ± 0.2*
	(18.9 ± 3.3)	(22.6 ± 3.1)	(20.2 ± 2.0)	(20.2 ± 0.7)
	[1.184 ± 0.034]	[1.524 ± 0.024]*	[0.898 ± 0.033]	[1.312 ± 0.056]*
Serum	-	-	-	-
	-	-	-	-
	[0.421 ± 0.047]	[0.571 ± 0.057]*	[0.519 ± 0.037]	[0.599 ± 0.047]
Placenta	7.6 ± 1.3	8.0 ± 1.5	9.0 ± 1.3	8.7 ± 2.3
	-	-	-	-
	[0.658 ± 0.097]	[0.724 ± 0.083]	[0.726 ± 0.087]	[0.803 ± 0.057]

[a]The content expressed as % of body burden to the whole fetus (= 100%) is shown in parenthesis. [b] The content expressed as ng TCDD/g tissue or ml serum is shown in brackets. Each value represents the mean ± S.D. of 3 parents: the data for all fetuses in one parent are averaged to be one analyzing unit. * Significantly different from the value obtained one day after TCDD treatment ($P < 0.05$).

although the sensitivity to TCDD damage may not differ markedly between fetal and adult brain. Our observation reported here may support this view. However, when the brain content (ng/g tissue) of TCDD in fetuses of dams treated with TCDD was compared with those of the dams and TCDD-treated adult males, the values were comparable (see Tables 1 and 2). This seems to be due to the difference in the higher translocation to the brain + low body burden (fetuses) and lower translocation to the brain + high body burden (adults). In addition, we have previously shown that the intraventricular injection of TCDD into young-adult male rats does not cause any reduction in the expression of pituitary LHβ and FSHβ mRNAs, and testicular StAR mRNA (Takeda *et al.,* 2009). In that study, the amount of TCDD injected intraventricularly (10 μg/kg body weight) was extremely high (approximately 100,000 times) compared with the amount distributed to fetal brain following oral administration of 10 μg/kg TCDD to the dam (Table 2). The above pieces of evidence will never support the view that the easier translocation of TCDD to fetal brain is a determinant of fetus-specific damage to the syntheses of gonadotropins and steroidogenic proteins. Therefore, factor(s) apart for the difference in the distribution of TCDD to the brain would contribute to the occurrence of fetus-specific damage to steroidogenic systems.

One of the reasons for the easy translocation of TCDD to the brain in fetuses may be rationalized from the low ability of their peripheral tissues to accumulate dioxin. Liver is a representative tissue that accumulates TCDD. CYP1A2, a dioxin-inducible enzyme, is thought to play a major role as a TCDD reservoir, because of its high affinity for dioxins (Kuroki *et al.,* 1986; Smith *et al.,* 2001; Dragin *et al.,* 2006). Immunoblotting analysis has demon-

strated that hepatic CYP1A2 is not detectable in the fetal stages of rats (Elbarbry *et al*., 2007). This enzyme emerges at PND3 (approximately 25% of adult level), and its expression rises gradually to a maximum by early puberty (PND42) (Elbarbry *et al*., 2007). Therefore, it would be reasonable that the amount of TCDD delivered to the fetal brain becomes greater than in the case of adults, because of the absence of hepatic CYP1A2. Our observation that the TCDD content of fetal liver expressed as ng TCDD/g tissue was extremely low (only 2-6% of adult liver) agrees with above consideration.

This study was unable to clarify the mechanism underlying a fetus-specific reduction by TCDD in gonadotropin synthesis. As mentioned before, gonadotropin production in adult rats is resistant to TCDD, even although the high dose of the dioxin is directly injected into their brains. Gonadotropin production may be regulated by mechanisms which are different in fetuses and adults, and only the fetal mechanism may be damaged by TCDD. Further studies are required to clarify this possibility.

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