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Identification of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-inducible and -suppressive Genes in the Rat Placenta: Induction of Interferon-regulated Genes with Possible Inhibitory Roles for Angiogenesis in the Placenta

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Abstract. Exposure to a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) results in a variety of toxic manifestations, including fetal death. In order to evaluate the effects of low dose TCDD on placental function, pregnant Holtzman rats were given a single oral dose of 1600 ng TCDD/kg body wt or an equivalent volume of vehicle (control) on gestation day 15 (GD15), and changes in the gene expression in the placenta on GD20 were analyzed by two comprehensive methods, representational difference analysis (RDA) and DNA microarray technology. Candidates of TCDD-inducible and -suppressive genes were selected. Quantitative real-time PCR analysis was then performed to verify the induction or suppression levels of the candidate genes. Finally, we identified 81 TCDD-inducible and 21 TCDD-suppressive genes from the placenta of TCDD-treated Holtzman rats on GD20. One of the remarkable profiles of the gene expression was that glucose transporters were strongly up-regulated by the TCDD treatment. Furthermore, many interferon-inducible genes were also up-regulated by the treatment. They included several cytokines such as IP-10 known as a potent angiogenesis inhibitor. In addition, interferon molecules are known to suppress angiogenesis. The above observations suggest that activation of the interferon signaling pathway and the induction of anti-angiogenic factors by TCDD might have a role in causing the inhibition of neovascularization, resulting in the hypoxic state of placenta and increased incidence of fetal death.

Key words: TCDD, Placenta, Gene expression, Real-time PCR, DNA microarray

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is known to be the most toxic congener among the dioxin and related compounds found in the environment. Exposure to TCDD causes a diverse spectrum of toxicities in humans and laboratory animals [1–4]. The fetus is one of the most sensitive targets of TCDD and exhibits a wide range of biological responses at low TCDD levels that have no detectable effects on maternal side (usually the levels were ten to hundred times lower than those of LD50). One of the most severe adverse effects of TCDD is intrauterine fetal death [1, 5–7]. The incidence has been shown in all species studied to date, including the monkey, hamster, rat, and mouse. Although fetal death is an important aspect of TCDD toxicity, its precise mechanism is not well understood. Placenta plays a crucial role in maintaining normal fetal growth such as exchange of oxygen and carbohydrate nutrients. In previous studies Ishimura *et al.*

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demonstrated that exposure of pregnant rats to 800 or 1600 ng TCDD/kg on gestational day 15 (GD15) resulted in an increased incidence of fetal death on GD20 [8, 9]. In their experimental protocol, the placenta showed several abnormalities that led to increased risk for fetal death. Exposure to TCDD altered the glucose kinetics in placenta [8] and caused reduced blood flow and placental hypoxia [9], leading them to hypothesize that the increased incidence of the fetal death may be due to reduced blood flow into the placenta [8, 9]. In order to clarify what kind of genes are involved in these placental abnormalities or what other aspects of TCDD toxicities exist in the placenta, we conducted a comprehensive analysis of gene expression in placenta of pregnant Holtzman rats treated with TCDD using representational difference analysis (RDA) and DNA microarray technology. RDA is a subtraction cloning method developed by Pastorian *et al.* [10]. The RDA and the DNA microarray are very powerful and comprehensive methods to identify inducible or suppressive genes by given hormonal or pharmacological treatments. Previously we have reported many gonadotropin-inducible genes in the rat ovary by using those methods [11–13]. In this study, many candidates for placental TCDD-inducible or -suppressive genes were selected by those methods, and the induction or suppression was verified by quantitative real-time PCR.

Profiling analysis of the verified genes revealed that, in addition to the genes involved in the glucose uptake, those involved in the interferon signaling pathway were strongly induced by the TCDD treatment. Based on the results obtained, the molecular mechanisms of placental disorders by low dose TCDD will be discussed.

Materials and Methods

Reagents

2,3,7,8-TCDD was obtained from Cambridge Isotope Laboratory (Andover, MA). Rat cDNA glass array (Gene Chip Rat Expression Array 230A) was from Affymetrix, Inc., Santa Clara, CA.

Animals

Holtzman rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The animals were maintained in a controlled environment with 12/12 h light/dark cycles, and given free access to laboratory rat chow and water. Female rats were allowed to mate on proestrus overnight, and, if vaginal plugs were observed in the morning, the day was designated GD0. Six pregnant rats were treated with TCDD, and a total of 10 dead fetuses was observed among 83 fetuses. The pregnant rats were housed individually until exercised. All animal experiments were performed according to the guidelines for animal welfare of the National Institute for Environmental Studies [8, 9].

TCDD treatment

The pregnant animals were exposed to TCDD as described previously [8, 9]. TCDD was dissolved in nonane at a concentration of 20 μg/ml, and the solution was diluted with corn oil. On GD15 the pregnant rats were given a single oral dose of 1600 ng TCDD/kg body wt or an equivalent volume of mixture of nonane and corn oil solution (control). Placentas were collected from the TCDD treated or control rats on GD20. The placental samples were immediately frozen in liquid nitrogen and maintained at −80°C until used.

Representational difference analysis

For isolating TCDD-inducible and -suppressive genes from the placenta, representational difference analysis (RDA) was performed according to the procedure reported by Pastorian *et al.* [10]. Briefly, total RNA was extracted from each placenta. Each five placental RNA samples were mixed and used for further analysis. After Oligo-dT latex beads treatment, mRNA preparations were then converted to respective double-stranded cDNAs. Tester TCDD-treated placental cDNAs and driver control placental cDNAs were digested with a restriction enzyme DpnII. The digested tester cDNA fragments were ligated with first-round adaptor oligonucleotide molecules, and then mixed with the excess amount of the enzyme digested driver cDNAs. The mixture was denatured and then incubated at 67°C for 16 hours for hybridization. After the hybridization, only the tester specific cDNA fragments were amplified by PCR reaction using primers complementary to the adaptor sequence. For the second cycle RDA, the resulting PCR products, in which the tester specific cDNA fragments were enriched, were digested again with DpnII, and then followed by the second adaptor

ligation and hybridization in order to further enrich tester specific cDNA fragments. The PCR products after the second RDA reaction were ligated in to pGEM T-vector, and individual clones were analyzed to identify TCDD-sensitive genes.

DNA microarray

The microarray method was carried out according to the manufacturer's instruction.

Briefly, total RNA was extracted from the TCDD-treated and control placentas described above. Double stranded cDNA libraries were constructed from the mRNA of TCDD-treated and the control placentas using an oligo-dT primer with a T7-promoter sequence at the 5'-end. Biotin-labeled complementary RNA was *in vitro* transcribed by T7 polymerase using the cDNA libraries as template. The biotin-labeled RNA was fragmented and hybridized to the Rat cDNA glass array for 16 hr at 45°C and then washed and stained using the GeneChip Fluidics. The array was scanned by a Gene-Array scanner, and hybridization patterns were detected as fluorescent light from reporter groups that have been incorporated into the target genes. The average difference measurements computed in the Affymetrix Microarray Analysis Suite 4.0 serve as a relative indicator of the level of expression.

Quantitative Real-Time PCR

Messenger RNA was extracted using an RNA extraction solution (Trizol) and oligo-dT latex beads as described previously [11–13]. Five micrograms of mRNA preparations were reverse-transcribed, and then converted to double stranded cDNA molecules. Complementary DNA was quantified by UV absorption measurement, and 1 ng was subjected to the PCR reaction as template. As an internal standard, TATA binding protein (TBP) was used instead of GAPDH, since GAPDH gene expression was affected by the TCDD treatment (data not shown). PCR reaction involved template cDNA samples, Advantage Taq Plus DNA polymerase (Clontech), dNTP, and Syber Green. Serial dilutions of the templates were used to create a concentration curve, and relative expression levels were calculated for each sample [14]. Abundance of each gene was referred to as a Ct (cycle threshold) value in this system.

Results

Exposure of pregnant Holtzman rats to 1600 ng TCDD/kg on GD15 resulted in an increased incidence of fetal death on GD20 [8, 9]. In these studies, each placenta from the TCDD-treated and control rats was prepared by the same exposure protocol as previous studies [8, 9], and gene expression in the placenta was analyzed by RDA and DNA microarray methods. Generally the RDA method is more sensitive than the DNA microarray, while the latter covers more comprehensive genes than the RDA method. A total of 2536 clones were picked-up and characterized from the RDA-subtracted cDNA libraries as candidate genes. In addition, the same tissues were used for the DNA-microarray, and among 13000 genes spotted on the array, 43 TCDD-inducible and 18 TCDD-suppressive candidate genes (cut-off values of 2.0 as inducible and 0.5 as suppressive genes) were also picked-up. All of these candidate genes picked-up by both methods were verified by using real-time PCR analysis, and genes that showed expression ratios (TCDD-treated/control) of more than 2.0 or less than 0.5 were finally identified as TCDD-sensitive. TCDD-inducible and -suppressive genes in the rat placenta identified by RDA and DNA microarray methods were summarized in Table 1 and Table 2, respectively.

TCDD-inducible genes in the placenta

As listed in Table 1, 81 genes were identified as TCDD-inducible genes in the placenta, 22 genes of which were from the results of DNA microarray analysis. Eleven genes were reported only on EST databases, and one gene showed no significant similarity to any gene on DNA databases. Other genes were all annotated as shown in Table 1 including those homologues of human or mouse. They were categorized into several groups. Enzyme genes include CYP1A1 and CYP1B1 that are known as the typical TCDD target genes [15, 16]. Inducible genes in placenta include some of the major blood proteins, such as prealbumin, apolipoproteins, transferrin, retinol-binding protein, prothrombin, and fibrinogens. This suggests that the placental production of the blood proteins was stimulated by the TCDD treatment. In addition, two placental specific genes, alpha-fetoprotein and pregnancy-specific glycoprotein (mCGM3), were also induced by the TCDD treatment.

Table 1. TCDD-inducible genes in the rat placenta

Molecular function	Description	Array ratio	Real Time PCR ratio	Ct	Accession
Enzyme	(LMW) K-kininogen	3.32	8.14	30.06	M11884
	(LMW) T-kininogen I	ND	4.43	30.53	M11883
	Aldolase B	ND	2.72	34.15	M10149
	Alpha-1-protease inhibitor	ND	3.37	32.17	D00675
	Alpha-2 antiplasmin	ND	3.38	30.03	AY216659
	Alpha-fibrinogen	5.77	5.05	33.13	M35601
	Brain 4.1(L)	ND	2.24	29.48	AB019257
	CYP1A1	ND	425.81	26.13	K02246
	CYP1B1	ND	3.90	34.60	X83867
	Cathepsin B	ND	2.11	19.33	X82396
	Creatine kinase-B	ND	2.62	29.12	M57664
	Cytosolic NADP-dependent isocitrate dehydrogenase	ND	3.77	24.51	L35317
	Fibrinogen B-beta-chain	5.87	7.95	30.89	M27220
	Glutathione S-transferase Ya subunit	ND	4.27	31.73	M26874
	NADH dehydrogenase (ubiquinone) Fe-S protein 7	ND	2.99	26.11	BC013503
	NC1 protein (nc1 gene)	ND	2.25	28.63	AJ250730
	Peroxisomal enoyl hydratase-like protein (PXEL)	ND	2.01	22.38	U08976
	Protein C	3.91	4.10	29.36	X64336
	Prothrombin precursor (F2 gene)	4.38	5.92	24.44	X52835
	RASP1	ND	2.52	31.93	U55765
	Stearoyl-CoA desaturase 2	ND	2.31	29.98	AB032243
	Tissue factor protein	1.93	2.29	25.09	U07619
Transcription Factor	Zinc finger homeodomain enhancer-binding protein-1 (Zfhep-1)	ND	2.36	32.92	U51583
Immunity Protein	Da1-24	3.17	2.64	—	AY325253
	Interleukin-12 p40 precursor	ND	1.96	35.40	AF133197
	MHC class I antigen (RT1-E gene)	ND	2.42	—	AJ306619
	MHC class I protein	ND	6.46	—	L26224
	Pregnancy-specific glycoprotein (mCGM3)	ND	2.17	—	U09815
Signal Transducer	Beta ig-h3	ND	2.73	31.93	AF305713
	CXC chemokine LIX	ND	2.60	31.90	U90448
	Inhibin alpha-subunit	ND	1.98	27.41	M36453
	Interferon beta	ND	5.07	36.92	D87919
	Interferon inducible protein 10 (IP-10)	5.03	9.48	25.38	U22520
	Macrophage inflammatory protein-2	ND	6.29	32.48	AB060092
	Monokine induced by interferon gamma (Mig)	ND	3.28	33.97	AF537208
	Proliferin-related protein	1.94	1.82	24.23	AF139809
Transporter	Alpha-fetoprotein (AFP)	ND	5.47	27.57	X02361
	ApolipoproteinA-I	ND	6.26	26.66	M00001
	Apolipoprotein A-IV	5.07	4.24	21.71	M00002
	Apolipoprotein B	3.36	7.07	28.26	M27440
	Beta-2 glycoprotein I	5.10	6.28	30.95	X15551
	Beta-globin	ND	3.71	13.83	X16417
	GLUT2	ND	7.39	31.47	J03145
	GLUT4	ND	2.84	31.49	D28561
	Retinol-binding protein (RBP)	2.75	5.39	32.55	M10934
	Transferrin	5.89	4.12	22.82	X77158
	Transferrin-like	ND	4.08	26.01	AF476964
Structural Protein and Other Groups	Angiopoietin-related protein 3	ND	8.90	30.40	*CB581433
	Claudin 2	2.29	3.27	26.96	*BM392116
	Collagen alpha 1 type XI	ND	2.60	33.72	AJ005396
	Collagen type XXVII proalpha 1 chain (col27a1)	ND	1.98	28.20	AY232999

Table 1. (continued)

Molecular function	Description	Array ratio	Real Time PCR ratio	Ct	Accession
	Decorin	ND	2.07	24.20	X59859
	Ficolin-B	ND	2.33	29.23	AB036792
	Glucocorticoid-attenuated response gene 16	ND	92.29	27.14	AJ276893
	Glucocorticoid-attenuated response gene 39	ND	5.54	28.07	*CB719539
	Hypothetical protein RMT-7	ND	2.09	23.13	AF465614
	Interferon-inducible protein homologue	ND	6.15	24.96	*CB610262
	Interferon, alpha-inducible protein (G1p2)	ND	2.76	23.17	*CB790136
	Matrix metalloproteinase inhibitor (TIMP-1)	2.15	2.11	25.20	L31883
	Mx1	4.51	6.41	26.00	X52711
	Mx2	3.80	7.27	25.71	X52712
	Mx3	ND	3.06	26.70	X52713
	Prealbumin	2.04	4.76	27.03	K03252
	Pro alpha 1 collagen type III	ND	2.56	26.78	X70369
	Proteasome subunit R-RING12	ND	2.92	27.78	D10757
	Ribosomal protein L5	ND	2.34	20.59	X06148
	Similar to PC-LKC gene product	1.92	2.06	27.53	*BI291884
	Similar to vinculin	ND	1.99	27.73	*CB757866
	Spp-24 precursor	3.03	6.13	25.56	U19485
	TORID	ND	2.53	25.27	AF370882
	Thrombospondin	ND	2.30	28.72	*AB113080
	Type VI collagen alpha3 subunit	1.92	1.94	25.49	*AI176126
Function Unknown	AB113071	ND	2.37	31.85	*AB113071
	Clone nrhy5-00111-g2	ND	3.17	27.96	*CB546450
	DRNBNB02	ND	2.06	24.98	*BG671212
	EST196998	1.98	2.16	30.78	*AA893195
	LRRGT00077	ND	2.29	30.17	*AY387063
	UI-R-BJ0p-afw-e-10-0-UI.r1	ND	2.14	27.92	*BF566943
	UI-R-C4-akz-c-07-0-UI.s1	3.22	2.99	24.70	*AW531805
	UI-R-E0-bq-f-06-0-UI.r1	ND	5.64	30.34	*BF550478
	Unknown	ND	2.05	26.30	ND

ND: Not determined, Ct: cycle threshold, *: Rat EST database

Two characteristic profiles of the induction were (1) glucose transporters and (2) interferon-related genes, details of which will be described elsewhere.

TCDD-suppressive genes in the placenta

As listed in Table 2, 21 genes were identified as TCDD-suppressive genes in the placenta, 10 genes of which were from the results of the DNA microarray analysis. Six genes were reported only on EST databases, and three genes showed no significant similarity to any gene on existing DNA databases. Other genes were all annotated as shown in Table 2 including those homologues of human or mouse.

Induction of glucose transporter family genes

As shown in Fig. 1, expressions of four major glucose transporter genes were examined. Quantitative real-time PCR revealed that GLUT2 and GLUT4 were strongly induced by the TCDD treatment in the placenta. Gene expression of GLUT3 was also marginally increased by the treatment (1.58-fold), but that of GLUT1 was less affected (1.24-fold). In the control placenta, GLUT3 was abundantly expressed (Ct26.57), while expression levels of the other three transporter genes were relatively low (Ct32.73 for GLUT1, Ct31.47 for GLUT2, and Ct31.49 for GLUT4). Therefore, in addition to the remarkable induction of GLUT2 and GLUT4 genes, a small but significant increase in GLUT3 gene expression may have a major physiolog-

Table 2. TCDD-suppressive genes in the rat placenta

Molecular function	Description	Array ratio	Real Time PCR ratio	Ct	Accession
Enzyme	Urokinase-type plasminogen activator	0.48	0.45	27.99	X63434
	LASP-1	ND	0.19	25.46	AF242187
Signal Transducer	Hepatic product spot 14	0.43	0.33	31.78	K01934
	Prepronociceptin	0.59	0.63	25.84	X97375
	Prolactin-like protein H	0.50	0.41	27.74	AB009889
Transcription Factor	Transcription factor GATA-1	0.55	0.47	31.50	D13518
	Zinc finger protein 52	ND	0.16	34.17	*CK470357
Transporter	mBLVR	ND	0.54	25.50	*CB557112
Structural Protein and Other Groups	Talin	ND	0.35	26.13	*CK366133
Function Unknown	Ab2-225	0.56	0.60	27.78	AY325197
	BB857172	ND	0.16	29.50	*BB857172
	Clone mrpe4-00034-a12	ND	0.16	32.27	*CB760299
	Eker rat-associated intracisternal-A	ND	0.43	32.27	U23776
	EST224029	0.59	0.62	24.96	*AI180286
	FAD104	ND	0.49	25.21	*CB702955
	Nuclear-localized inactive X-specific transcript (Xist)	0.27	0.33	27.18	*AI228978
	UI-R-BJ1-auz-g-11-0-UI.s1	ND	0.31	25.56	*BE111117
	UI-R-CX0s-cct-f-09-0-UI.s1	0.43	0.54	27.16	*BI284907
	Unknown mRNA	0.43	0.48	26.53	AF152002
	Unknown	ND	0.45	31.31	ND
	Unknown	ND	0.51	24.21	ND

ND: Not determined, Ct: Cycle threshold, *: Rat EST database

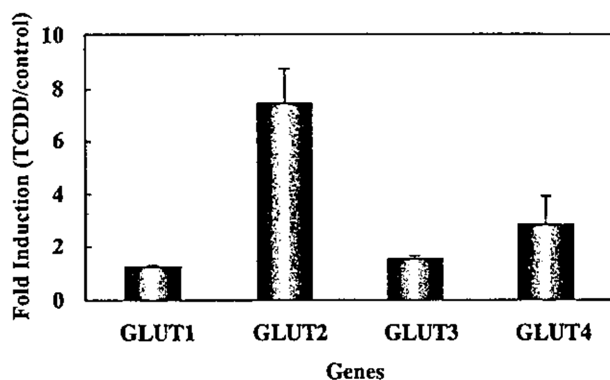

Fig. 1. Induction of glucose transporter genes in the rat placenta by TCDD.
Expression levels of major 4 glucose transporter genes were determined by quantitative real-time PCR, and changes in the expression levels were compared. Values were expressed as fold induction (TCDD/control). Serial dilutions of five points for each sample were used to make each dilution curve. TATA binding protein (TBP) was used as an internal standard to ensure equal loading of template cDNA molecules.

ical significance of the glucose-uptake in the TCDD-treated placenta.

Induction of interferon-related genes

One of the most remarkable findings of the pesent experiment was that many interferon-regulated genes were induced in the placenta of TCDD-treated animals. As shown in Fig. 2, 15 out of 18 interferon-inducible genes examined were strongly up-regulated by the TCDD treatment. Among them were interferon-inducible cytokines, such as IP-10 [17, 18], macrophage inflammatory protein-2, monokine induced by gamma interferon (Mig) [19] and CXC chemokine LIX [20]. Of these, IP-10 was reported to be involved in the interferon-mediated inhibition of angiogenesis [17, 18]. Since many interferon-inducible genes were up-regulated by the TCDD-treatment, we examined the levels of interferon family genes. At the transcription levels, the interferon beta gene was up-regulated (5.07-fold induction) in the TCDD-treated placenta (Table. 1), but we were not able to detect interferon-alpha and -gamma genes in our system (data not shown).

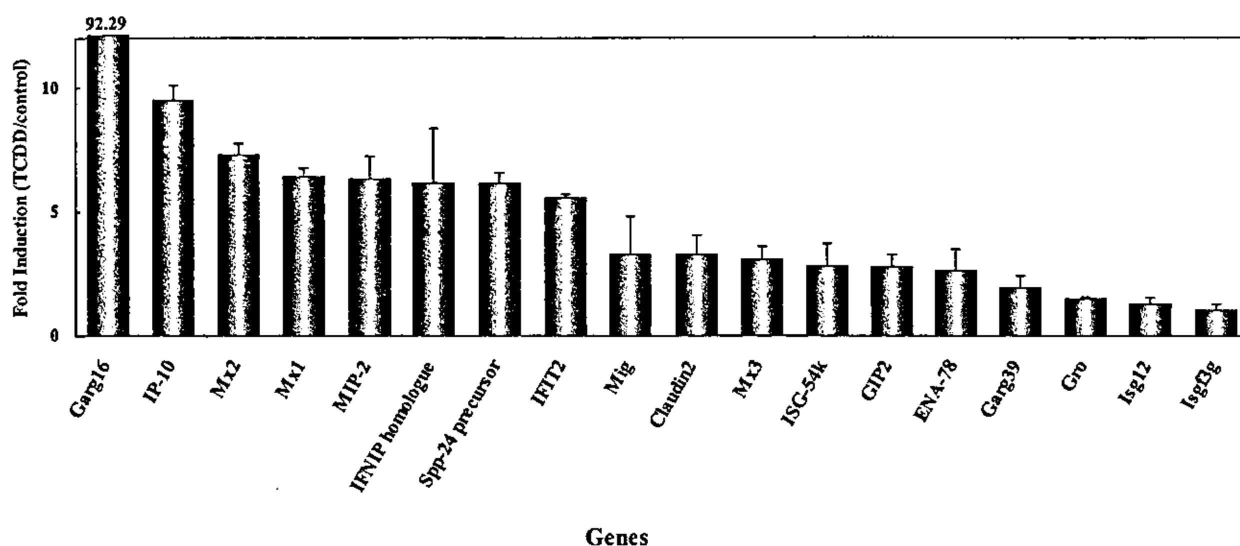

Fig. 2. Induction of interferon regulated genes in the rat placenta by TCDD.
Expression levels of 18 interferon regulated genes were determined by quantitative real-time PCR, and changes in the expression levels were compared. Values were expressed as fold induction (TCDD/control). Serial dilutions of five points for each sample were used to make each dilution curve. TATA binding protein (TBP) was used as an internal standard to ensure equal loading of template cDNA molecules.

Discussion

Previous studies have shown that a very low dose of TCDD results in the increased incidence of fetal death [8, 9]. Histological and protein profiling analysis revealed that the fetal death after the TCDD treatment may be due to the placental hypoxia [9]. In order to evaluate further the mechanisms of the induction of such the adverse effect, comprehensive analysis was done to examine changes in the placental gene expression. As clearly shown in Fig. 1, glucose transporter genes were all induced after the TCDD treatment. Ishimura *et al.* previously reported the histopathological changes in glycogen cells and the elevated levels of glucose content and GLUT3 mRNA expression in the placenta of the TCDD-exposed rats in comparison to those of the control rats [9]. In addition to GLUT3 mRNA, we showed here that GLUT2 and GLUT4 mRNAs were strongly induced by the TCDD-treatment, and that GLUT1 mRNA was less affected by the treatment, indicating that most of major glucose transporter genes were up-regulated by TCDD, which may lead to glycogen accumulation in the placenta. In our quantitative real-time PCR system, values of cycle threshold (Ct) represent expression levels of genes. The Ct value of GLUT3 mRNA was much lower than those of other GLUT family genes, suggesting that the major glucose transporter working in the placenta may be GLUT3, though strong induction of GLUT2 and GLUT4 genes may also play significant roles in the glucose accumulation in the TCDD-treated placenta. As far as we know, this is the first report describing that GLUT2 and GLUT4 genes were actually expressed in the placenta under specific conditions such as the TCDD treatment. It is likely that a tissue-specific responsive mechanism of TCDD exists for the induction of glucose transporters in the placenta, since those transporter genes were not up-regulated in other tissues such as the ovary from the same TCDD-treated animals (data not shown). Histological studies by Ishimura *et al.* showed that blood vessel formation in the placenta was severely impaired by the TCDD treatment (unpublished data), indicating that the angiogenesis was inhibited in the placentas of TCDD-treated animals on GD20. Therefore, the TCDD treatment may cause hypoxic conditions in placenta. It was reported that the expression of GLUT-1 and GLUT-3 mRNAs was up-regulated under hypoxic conditions [21].

Most striking observation of this experiment was that many interferon-inducible genes were up-regulated in the placenta of TCDD-treated animals. As shown in Fig. 2, 15 out of 18 interferon-inducible genes examined were induced by the TCDD treatment, which strongly suggests that interferon signaling pathway

[22] was activated by the TCDD treatment. We also examined the expression levels of interferon-alpha, -beta, and -gamma by the real-time PCR analysis. Only interferon-beta gene expression was detected, and the level was strongly up-regulated by the TCDD treatment. Although the data suggest that interferon-beta is the key factor, we must take into consideration of the fact that the interferon signaling pathway is activated by many other signaling molecules [23]. The typical interferon signaling pathway is activated through the JAK-STAT system [22]. It is well known that pituitary hormone GH and prolactin also activate the JAK-STAT system [23], and that several prolactin-like molecules including placental lactogen are abundantly produced in the placenta [24]. We can not exclude the possibility that, in addition to interferon-beta, the prolactin-like molecules produced in the placenta may also play significant roles in the induction of interferon-regulated genes.

It is noteworthy that interferon is known to be involved in the regulation of angiogenesis. Interferon-alpha and -gamma were reported to inhibit endothelial cell proliferation [25]. IP-10, one of the interferon-inducible genes, was also strongly induced in the TCDD-treated rat placenta. IP-10 is a member of the alpha-chemokine family, inhibits bone marrow colony formation, has anti-tumor activity *in vivo*, is chemoattractant for monocytes and T cells, and promotes T cell adhesion to endothelial cells. In addition to the above functions, IP-10 has been reported to be a potent inhibitor of angiogenesis *in vivo* [17, 18]. Furthermore, thrombospondin [26] and tissue inhibitor of metalloproteinase 1 (TIMP-1) [27] were also induced by the TCDD treatment, both of which are known to inhibit endothelial cell proliferation. Considering the above observations, the neovascularization in the placenta on GD20 may be impaired by the TCDD treatment through the induction of anti-angiogenic factors.

It is reasonable to speculate that the hypoxic state in the placentas of TCDD-treated rats may be due to the impairment of angiogenesis in the placenta, which may be caused by the activation of the interferon signaling pathway including the production of angiogenesis-inhibitory cytokine IP-10 as well as the production of anti-angiogenic factors such as thrombospondin and TIMP-1.

In conclusion, we identified 81 dioxin-inducible genes and 21 dioxin-suppressive genes from the placenta of TCDD-treated Holtzman rats on GD20. The present study revealed that glucose transporters were strongly up-regulated by the TCDD treatment.

In addition, many interferon-inducible genes were also up-regulated by the TCDD treatment, including IP-10, a potent angiogenesis-inhibitory cytokine. Activation of the interferon signaling pathway and the induction of anti-angiogenic factors may result in the hypoxic state of placenta, which may lead to the increased incidence of fetal death.

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Evaluation of relative potencies of PCB126 and PCB169 for the immunotoxicities in ovalbumin (OVA)-immunized mice

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Abstract

Dioxin-like polychlorinated biphenyls (PCBs) exert their toxicities by activating the arylhydrocarbon receptor (AhR), a ligand-dependent transcription factor, in a similar manner to the most toxic dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In the present study, we re-evaluated the relative potency (REP) of the toxic members of dioxin-like PCBs, PCB126 (toxic equivalency factor, TEF 0.1) and PCB169 (TEF 0.01) relative to TCDD, focusing our attention on their effects on the immune reactions of mice immunized with ovalbumin (OVA). Thymus involution, IgM production, and IL-5 produced by the splenocytes were examined in addition to CYP1A1 induction, the established index of AhR-activation, in the spleen. PCB126 had an REP value of 0.1 because of its effects on thymus, IgM, IL-5, and CYP1A1 induction in the spleen, although its effect on IgG1 production was weaker. On the other hand, PCB169 had a smaller REP value estimated at less than 0.01 with regard to CYP1A1 induction in the spleen and all examined immunological effects, except for IgM production. The tissue concentrations of PCB169 and TCDD could not explain the reason for the smaller potency of PCB169, since the spleen contained a higher proportion of PCB169 to TCDD than dosed. These results indicate that dioxin-like PCBs, especially PCB169, shows deviating REPs against immune reactions, and also suggest that PCB169-liganded AhR behaves differently from TCDD-liganded AhR in immune cells.
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Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD); PCB126; PCB169; Immunotoxicity; Toxic equivalency factor (TEF)

1. Introduction

Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlori-

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2. Materials and methods

2.1. Reagents

The TCDD with a purity of 98% was purchased from Cambridge Isotope Laboratory (Andover, MA). 3,3′,4,4′,5-Pentachlorobiphenyl (PCB126, higher than 99% purity) and 3,3′,4,4′,5,5′-hexachlorobiphenyl (PCB169, higher than 99% purity) were obtained from AccuStandard, Inc. (New Haven, CT). OVA (albumin, chicken egg, grade VII) was purchased from Sigma (St. Louis, MO), and for immunization, the alum-precipitated OVA was prepared as previously described (Inouye et al., 2003; Ito et al., 2002).

2.2. Animal treatments

Female C57BL/6J mice (5 weeks old) were purchased from Clea Japan (Tokyo) and acclimatized to the environment for 1 week prior to use. They were given food and distilled water ad libitum and were maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of 50 ± 10%, and a 12 h light:12 h dark cycle. These mice were handled in a humane manner following the NIES guidelines for animal experiments.

Five mice in individual groups were given single doses of TCDD or PCB in corn oil in a volume of 10.0 ml/kg body weight. Mice in the control group were given corn oil only. They were then immunized intraperitoneally with 100 μg of alum-precipitated OVA (Inouye et al. 2003; Ito et al., 2002). On specified days after immunization, the animals were sacrificed by cervical dislocation under a diethyl ether anesthesia, and the plasmas, thymuses, spleens and livers were examined.

2.3. Flow cytometric analysis

Percentages of the T and B cells in the spleen were measured by flow cytometry with FACSCalibur (Becton-Dickinson, Mountain View, CA) as described previously (Nohara et al., 2002). T and B cells were stained with phycoerythrin (PE)-labeled anti-mouse CD3 (17A2) and fluorescein isothiocyanate (FITC)-labeled anti-mouse CD45R/B220 (RA3-6B2), respectively. The percentages of $CD4^+$ and $CD8^+$ T cells in the thymocytes and splenocytes were determined by staining with PE-labeled anti-mouse CD4 (GK1.5) and FITC-labeled anti-mouse CD8 (53–6.7). All antibodies were purchased from PharMingen (San Diego, CA).

2.4. IL-5 measurement

Spleen cells were prepared on day 4 after administration and immunization, and were cultured at a 1×10^6 cells/200 μl complete medium with or without OVA (100 μg/ml) for 3 days (Nohara et al., 2002). The culture medium was separated and the IL-5 in the supernatant was measured using ENDOGEN Matched Antibody Pair (ENDOGEN, Woburn, MA) according to the manufacturer's instructions.

2.5. Antibody measurement

Plasma samples were prepared on day 10 after animal treatments. OVA-specific IgM and IgG1 in the plasma were determined by ELISA as previously described (Inouye et al., 2003).

2.6. RNA extraction and RT-PCR

The total RNA was extracted from the livers and spleens using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The purity of each RNA preparation was evaluated by the ratio of absorbance at 260–280 nm, and the integrity of the preparation was assessed by 1.2% agarose gel electrophoresis. For cDNA synthesis, 0.1 μg of total RNA was transcribed using an RNA AMV RT kit (TaKaRa, Otsu, Japan) following the manufacturer's instructions. The primers and thermal cycling condition for PCR amplification are described elsewhere (Doi et al., 2003). Hypoxanthine phosphoribosyl transferase (HPRT) was used as an internal control to standardize cDNA concentration. PCR cycles were titrated to document the linearity of the signal strength, and PCR products were quantified during the exponential phases of amplification. The total number of cycles for each gene was as follows: 24 for liver CYP1A1, 33 for spleen CYP1A1, 28 for liver HPRT and 26 for spleen HPRT.

2.7. Quantitative real-time RT-PCR

cDNAs prepared as described above were added to 25 μl reaction mixtures containing 12.5 μl of 2X SYBR Green master mix (Applied Biosystems,

Table 2
Thymocyte number and subpopulation in mice exposed to TCDD or PCB169

Group	Thymocytes ($\times 10^7$ cells)	Subpopulations (%)			
		DN[a]	DP[a]	CD4SP[a]	CD8SP[a]
Vehicle	10.4 ± 0.8	4.5 ± 0.4	84.4 ± 1.1	8.1 ± 0.4	4.6 ± 0.5
TCDD (20)	3.7 ± 0.4[b]	6.1 ± 0.5[c]	67.3 ± 1.1[b]	16.1 ± 0.4[b]	10.6 ± 0.5[b]
PCB169 (2000)	8.5 ± 1.2	5.4 ± 0.8	80.5 ± 2.0	8.4 ± 0.7	5.8 ± 0.7
PCB169 (5000)	7.3 ± 1.8	9.1 ± 2.0[c]	72.9 ± 3.5[c]	10.6 ± 1.2	7.4 ± 0.7[c]

Mice were given corn oil (vehicle), TCDD or PCB169 (the dose was indicated in parentheses in μg/kg body weight) and subsequently immunized with OVA/alum. The thymocytes were examined 4 days after the treatment.
[a] DN, double negative cells; DP, double positive cells; SP, single positive cells.
[b] $P < 0.01$.
[c] $P < 0.05$.

thymocyte number or DP cell percentage, and did not cause significant increase in CD8 single positive cell percentage, which was observed in the case of TCDD exposure. Upon exposure to 5000 μg PCB169/kg, the ratio of DP cells and CD8 single positive cells were changed, while the thymocyte number was not significantly affected. The effects of TCDD on these endpoints were consistently observed in the two sets of experiments as shown in Figs. 1 and 4 and Tables 1 and 2.

IgM production was similarly suppressed by TCDD (20 μg/kg) and PCB169 (2000 or 5000 μg/kg), while IgG1 was not suppressed by PCB169 at either dose (Fig. 5). These features are similar to those found with PCB126. Comparing it to PCB126, PCB169 did not efficiently suppress IL-5 production (Fig. 6). The effects of 2000 μg PCB169/kg on all the endpoints were confirmed in another independent experiment (data not shown). TCDD again reproducibly affected all of these endpoints in the two sets of experiments as shown in Figs. 2, 3, 5 and 6.

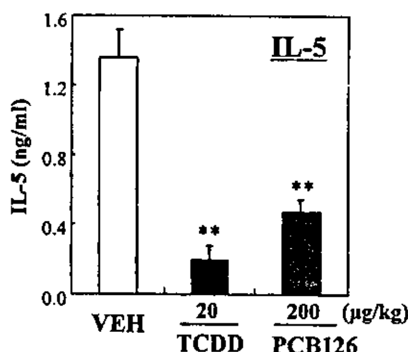

Fig. 3. Effect of TCDD- or PCB126-exposure on the interleukin-5 (IL-5) production by the splenocytes re-stimulated with OVA ex vivo. Mice were treated as described in the legend for Fig. 1. Four days after the treatments, the splenocytes were prepared and equal numbers of cells were cultured with OVA for 72 h. IL-5 in the supernatant was measured by ELISA. Results are presented as means ± S.E. ($n = 5$). $^{**}P < 0.01$.

3.3. CYP1A1 induction by TCDD and PCBs in the spleen and liver

Since the potency of PCB169 in the thymus changes and IL-5 production was found to be less than expected from the TEF, CYP1A1 induction, a direct target of AhR-activation, was examined in the spleen in comparison with the liver. PCB126 induced CYP1A1 expression in the spleen and liver at the same level as TCDD (Fig. 7a), as expected from the data on thymus atrophy and liver weight increase (Fig. 1). On the other hand, the potency of PCB169 (2000 or 5000 μg/kg) to induce CYP1A1 expression was much less than that of TCDD in the spleen, albeit similar in the liver (Fig. 7b and c).

3.4. Distribution of TCDD and PCB169 in the liver and spleen

Seeking an explanation for the weaker potency of PCB169 in CYP1A1 induction in the spleen than in the liver, we measured the concentrations of TCDD and PCB169 in these organs on day 4 after administration. As compared in Table 3, when TCDD (20 μg/kg) and PCB169 (2000 or 5000 μg/kg) were administered in a ratio of 1:100:250 by weight, distribution of these compounds in the liver were parallel to the doses. In the spleen of the mice dosed with 2000 μg PCB169/kg, a

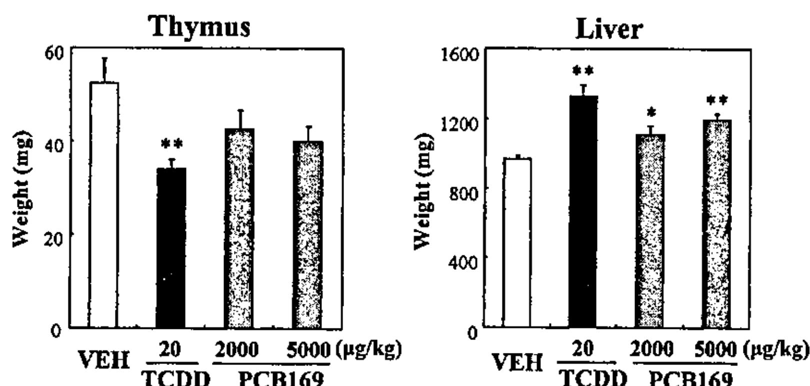

Fig. 4. Thymus and liver weight in the mice exposed to TCDD or PCB169. Mice were given corn oil (vehicle, VEH), TCDD or PCB169 and subsequently immunized with OVA/alum. The thymus and liver weights were measured 4 days after the treatments. Results are presented as means ± S.E. ($n = 5$). *$P < 0.05$, **$P < 0.01$.

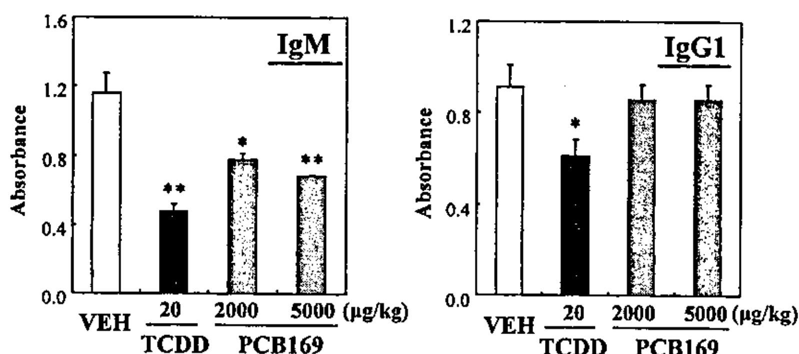

Fig. 5. Effect of TCDD- or PCB169-exposure on the OVA-specific antibody production. Mice were treated as described in the legend for Fig. 4. Ten days after the treatments, OVA-specific IgM and IgG1 antibodies in the plasma were measured by ELISA. Results are presented as mean ± S.E. ($n = 5$). *$P < 0.05$, **$P < 0.01$.

relatively larger amount of the compound was accumulated (Table 3), showing that the distribution of PCB169 does not account for the weaker CYP1A1 induction in the spleen.

Table 3
Concentrations of TCDD and PCB169 in the liver and spleen on day 4 after treatment[a]

Group	Oral dose		Tissue distribution (ng/g)			
	μg/kg	Ratio	Liver[a]	Ratio	Spleen[a]	Ratio
TCDD	20	1	88.5	1	0.939	1
PCB169	2000	100	11100	125	304	324
PCB169	5000	250	27600	311	361	384

[a] Tissue distribution of each compound was measured with the combined samples from five animals per group.

4. Discussion

In the present study, we re-evaluated REPs in the immunotoxicity of PCB126 (TEF 0.1) and PCB169 (TEF 0.01), the toxic congeners of PCBs. In terms of the effect on liver weight increase, the REP for each congener was consistent with its TEF. The REP value of 0.1 for PCB126 was also supported by the effects on thymus, IgM, IL-5 and CYP1A1 induction in the spleen, although IgG1 production was less affected by PCB126. On the other hand, PCB169 had a smaller REP value estimated at less than 0.01 with regard to CYP1A1 induction in the apleen and all examined immunological effects, except for IgM production. Combining these results, we obtained two major characteristics of REPs of PCB126 and PCB169 from immunotoxicological

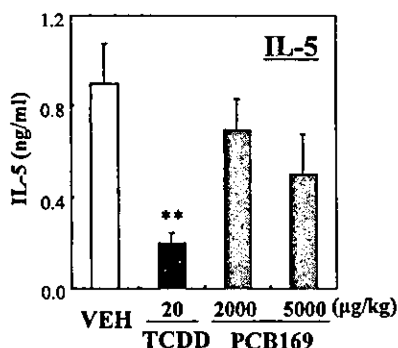

Fig. 6. Effect of TCDD- or PCB169-exposure on the interleukin-5 (IL-5) production by the splenocytes re-stimulated with OVA ex vivo. Mice were treated as described in the legend for Fig. 4. Four days after the treatments, the splenocytes were prepared and equal numbers of cells were cultured with OVA for 72 h. The IL-5 in the supernatant was measured by ELISA. Results are presented as mean ± S.E. ($n = 5$). $^{**}P < 0.01$.

aspects: (1) REP of PCB169 was smaller than 0.01 for several immunological endpoints we examined, and (2) REPs of PCB126 and PCB169 for IgM production were parallel to the TEF values, while the REPs of both PCBs for IgG1 production were smaller.

When seeking the reason why PCB169 is less potent for immune function, we can preclude the distribution of the compound in the body, since we observed that the spleen contained a higher proportion of PCB169 to TCDD than dosed. Nevertheless, the spleen dosed with PCB169 (2000 or 5000 μg/kg) showed weaker CYP1A1 induction than the one dosed with TCDD (20 μg/kg). A possible reason is the organ-specific ligand-dependency of AhR-function. Matikainen et al. (2001) reported that the promoter region of Bax contained two XREs which responded to AhR activated with 9,10-dimethylbenz[*a*]anthracene (DMBA) but not with TCDD. These ligand dependencies of Bax promoter were attributed to a peculiar nucleotide sequence flanking each of the core XRE sequences (5′-GCGTG-3′) (Matikainen et al., 2001). However, this mechanism cannot be applied to the organ-specific ligand-dependency of REP for PCB169, since the structures of genes are supposed to be identical in every organ. Alternatively, ligand-dependency of the estrogen receptor (ER) may be a good analogy. ER bound with estrogen induces transcription of target genes by associating with co-activator complexes, while the binding of ER with an antagonist tamoxifen recruits co-repressor complexes, and then results in inactivation (Yamamoto et al., 2001). Ligand-activated AhR/ARNT heterodimer has been found to interact with numerous nuclear proteins including basal transcription factors, co-activators or co-repressors, and other functional proteins (Swanson, 2002). The AhR liganded with TCDD and PCB169 may individually interact with a different set of nuclear proteins in the spleen because of the organ-dependent distribution of those proteins.

In a previous study, Mayura et al. (1993) examined the immunotoxicity of PCB126 and PCB169 in C57BL/6 mice by measuring the number of splenic antigen-specific IgM producing cells against sheep red blood cells (SRBCs) using a plaque-forming cell (PFC) assay. They reported that PCBs 126 and 169 demonstrated almost the same order of potency as TCDD. However, we found that the REPs of PCB126 and PCB169 on IgM production were parallel to individual TEFs 0.1 and 0.01. A possible cause of the difference between the results obtained in Mayura et al.'s study (1993) and the present study is the antigens. We immunized the mice with OVA, which is known to elicit production of antibodies including IgE (Fujimaki et al., 2002). On the other hand, Mayura et al. used a SRBC antigen, which effectively induces cellular immune reactions, such as delayed-type hypersensitivity, in addition to the production of IgM and IgG, but not IgE class antibodies (Uchida et al., 1994). These antigens probably activate different cellular populations into different conditions, where individual sets of transcription factors and functional proteins are activated and AhR bound with PCBs and TCDD may function in individual ways through interaction with those factors. Similarly, B cells that are differentiated into IgM- and IgG1-producing cells may respond to AhR liganded with TCDD and PCBs in different manners, respectively, which may cause the differences between the REPs of the PCB congeners for suppression of IgM and IgG1 production.

The present study showed that TCDD and dioxin-like PCBs act differently in some immunological endpoints, and that the difference could not be explained by the distribution of these compounds in the body. Further studies on the interaction of AhR liganded with TCDD or PCBs and transcription factors, co-activators, and functional proteins would help better understanding of the effects of dioxin-related compounds.

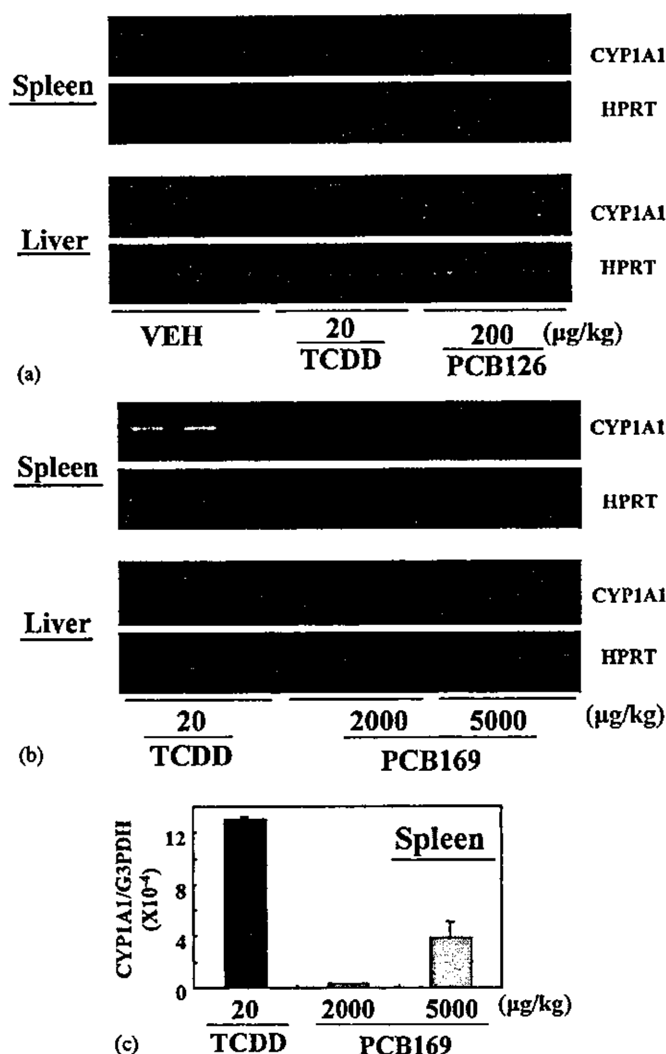

Fig. 7. Expression of CYP1A1 mRNA in the spleens and livers of TCDD- or PCB-exposed mice. Mice were given either corn oil (vehicle, VEH), TCDD, PCB126 or PCB169 and subsequently immunized with OVA/alum. Four days after the treatments, expression of CYP1A1 mRNA in the livers or spleens was measured by RT-PCR (a, b) or quantified by real-time RT-PCR (c) as described in Materials and methods. Each column and vertical bar in (c) represents the mean ± S.E. ($n = 5$).

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Testicular cytochrome P450scc and LHR as possible targets of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the mouse

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Abstract

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in adult animals has been reported to perturb the regulation of steroidogenesis in the testis, possibly by arylhydrocarbon receptor (AhR). To clarify how AhR is involved in the testicular steroidogenesis, we carried out comparative experiments using wild-type and AhR-null male mice that were intraperitoneally administered TCDD. The TCDD administration to wild-type mice showed significant reduction of P450scc and LHR in the testis, whereas the levels in the AhR-null mouse testis were unchanged. To compare anti-androgenic properties on hypothalamo–pituitary–gonadal (HPG) axis, estradiol-3-benzoate (EB), a synthetic estrogen agonist, was administered to mice, the expression of the *LHα/FSHα*, *LHβ*, *FSHβ* and *GnRHR* genes was severely impaired in the pituitary gland, in contrast to no observed effects in the TCDD-treated mice. In addition, the expression of the *LHR* gene was increased in the testis of the EB-treated mice. These observations suggest that the target of TCDD is different from that of EB on HPG axis and that TCDD treatment suppresses the *P450scc* and *LHR* genes in the testis in an AhR-dependent manner.
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Keywords: TCDD; P450scc; Intratesticular testosterone; LHR; AhR-null mouse; Estradiol

1. Introduction

A persistent environmental toxicant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which has a broad spectrum of toxicities, including hepatotoxicity, carcinogenicity, reproductive and developmental toxicity, exhibits a strong affinity toward the arylhydrocarbon receptor (AhR). The TCDD–AhR complex is translocated into the nucleus, forms a heterodimeric protein complex with Arnt, and then is able to recognize the xenobiotic responsive elements (XRE) (Matsushita et al., 1993). Analysis of AhR-null mice has demonstrated that most of the toxicity caused by TCDD is dependent on AhR (Fernandez-Salguero et al., 1996; Schmidt et al., 1996; Mimura et al., 1997; Hundeiker et al., 1999; Peters et al., 1999; Thurmond et al., 1999; Buchanan et al., 2000; Lin et al., 2002).

It has been reported that exposure to a relatively low-dose of TCDD affects male reproductive organs, such as retarded development of the urogenital complex with a decrease in weight of prostate gland and a shortening of anogenital distance of the offspring (Ohsako et al., 2001), and reduction of sperm number (Mably et al., 1992). The male reproductive disorder is one of the most sensitive endpoints for TCDD toxicity (Peterson et al., 1993; Gao et al., 1999). In particular, the reduction of sperm number is an important index for determining the tolerable daily intake (TDI) of dioxin and dioxin-related compounds.

Exposure to TCDD by adult rodents has been reported to result in the reduction of testosterone in the serum and testis (Moore et al., 1985; Mebus et al., 1987; Bookstaff et al.,

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1990a; Kleeman et al., 1990). When maternal exposure to TCDD affected production of testosterone in the juvenile, pubertal, or post-pubertal stage of offspring (Mably et al., 1992; Gray et al., 1997; Faqi et al., 1998), it is conceivable that regulation of testosterone production under these conditions may be perturbed at the critical target site(s) of TCDD.

The mechanism by which high-doses of TCDD impedes biosynthesis of testosterone is thought to be caused by an inhibition of the rate-limiting step of steroidgenesis in the testis, that is the cytochrome P450 side chain cleavage (P450scc) catalytic activity and the luteinizing hormone (LH)-stimulated mobilization of cholesterol to P450scc (Moore et al., 1985; Kleeman et al., 1990). However, whether the inhibition of P450scc activity by TCDD is accompanied by a reduction in the expression of *P450scc* or another related receptor gene is largely unknown. The critical target of TCDD in the testis might well be the expression of these genes in the Leydig cells.

When serum testosterone concentration is reduced, the responsiveness to gonadotropins-releasing hormone (GnRH) rises due to an increase in the number of GnRH receptors (GnRHR) followed by a subsequent rise in the synthesis and secretion of LH from the pituitary gland. The decrease in the serum testosterone concentration was found to be maximally developed 7 days after TCDD administration without an increase in the serum LH concentration (Moore et al., 1985, 1989). The mechanism of this imbalance between serum testosterone and LH concentrations has been studied by Peterson and co-workers (Bookstaff et al., 1990a). The levels of GnRH responsiveness and GnRHR molecules were lower in TCDD-treated rats, which were castrated and implanted with sustained-release low-dose of testosterone implants, than vehicle-treated rats. This suggested that these effects are due to an increase in the potency of testosterone (Bookstaff et al., 1990b). However, whether this potency is increased by TCDD in the non-castrated animals has yet to be examined.

In the present study, we investigated effects of TCDD on steroidogenesis in adult mice and further examined whether AhR is involved in the perturbation by TCDD by using AhR-null mice. In order to study how TCDD affects the regulation of testosterone production in vivo, we administered estradiol-3-benzoate (EB) as a synthetic estrogen agonist, which is known to affect the hypothalamo–pituitary axis, and compared the responses of the pituitary and testis between TCDD and estrogen agonist treatments.

2. Materials and methods

2.1. Chemicals and materials

2,3,7,8-TCDD (purity >99.5%) was purchased from Cambridge Isotope Laboratory (Andover, MA). Nonane, corn oil, EB (purity >99.0%) was obtained from Sigma (St. Louis, MO). Superscript™ II RNase H-Reverse Transcriptase, oligo(dT) 12–18 primer, and Ultrapure™ Agarose were purchased from Life Technologies (Rockville, MD, USA). Rabbit anti-rat cytochrome P450scc antibody (Cat. No. AB1244) and donkey anti-goat IgG, horseradish peroxidase (HRP) conjugated, species adsorbed for dual labeling secondary antibody (Cat. No. AP180P) were from Chemicon International (Temecula, CA, USA). Goat anti-rabbit IgG, HRP conjugated, species adsorbed for dual labeling secondary antibody and BCA protein assay reagent were from Pierce (Bezons, Cedex, France). Goat anti-mouse HSP60 antibody (Cat. No. sc-1052) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) Western blotting detection kit was from Amasham Pharmacia Biosciences (Buckinghamshire, UK). The QIA prep RNA purification kit (RNeasy) was from QIAGEN GmbH (Hilden, Germany). TaKaRa Ex Taq™ polymerase with 10 × Ex Taq™ buffer, TaKaRa LA Taq™ polymerase with 2 × GC buffer I, II, and 10 mM dNTP mixture were from TaKaRa Biomedicals (Otsu, Japan). The testosterone EIA kit was from Cayman Chemical Company (Ann Arbor, MI, USA). The SDS–PAGE gel mini was from TEFCO Corporation (Tokyo, Japan) and Immobilon™-P was from Millipore (Bedford, MA, USA).

2.2. Animals

Animals were handled with humane care according to the guidelines on animal experiments at NIES. In the first experiment, adult male C57BL/6J mice and AhR-null mice (C57BL/6J:129svJ:ICR = 2:1:1 mixed background) were dosed with 0.8, 4, 20 or 100 μg TCDD/kg body weight (bw) or vehicle (95% corn oil and 5% nonane). The mice were killed by cervical dislocation 7 days after administration, and their testes, adrenal and serum were collected. In the second experiment designed to observe pituitary response, adult male C57BL/6J mice were dosed with 100 μg TCDD/kg bw, 100 μg EB/kg bw or vehicle, and their testes, adrenal and pituitary were collected 7 days after administration. These samples were immediately frozen in liquid nitrogen and maintained at −80 °C until analyzed.

2.3. Semiquantitative RT-PCR

The semiquantitative RT-PCR method has been described elsewhere (Fukuzawa et al., 2003). Briefly, total RNA was purified from testis and adrenal using Trizol reagent. Total RNA from the pituitary was extracted with the QIA prep RNA purification kit. Total RNA (100 μg) was reverse-transcribed in a 20 μl reaction volume with 200 units of Superscript™ II reverse transcriptase and 0.5 μg of oligo (dT) 12–18 primer according to the standard protocol of the supplier. The reverse-transcribed samples were subjected to measurement of the mRNA levels, i.e., P450scc, steroidogeneic acute regulatory protein (StAR), GnRHR, a subunit of LH and FSH (LHα/FSHα), β subunit of LH (LHβ), β subunit of FSH (FSHβ), LH receptor (LHR),

Table 1
Primers used for semiquantitative RT-PCR

Genes	Primer sequence (5′–3′)		Product size (bp)	Tissue PCR cycle used		Annealing temperature (°C)	GenBank accession no.
	Forward primer	Reverse primer					
P450scc	CGCTCAGTGCTGGTCAAAG	GGTTGAGCATGGGGACACT	714	Testis	22	57	AF195119
				Adrenal	20	57	
StAR	CTCAACTGGAAGCAACACTC	CCGTGTCTTTTCCAATCCTC	308	Testis	24	56	AY032730
GnRHR	ATGGCTAACAATGCATC	ATATAACTGTGGTCCTG	528	Pituitary	26	50	NM010323
LHα/FSHα	ATGGATTACTACAGAAAAT	TGAAGTATAAGGGATGTAA	506	Pituitary	22	45	M22992
LHβ	TGCTGAGCCCAAGTGTGGT	GGAGGTCACAGGCCATTGG	363	Pituitary	23	60	U25145
FSHβ	AGCTGTTTACTTCCCAGACCATG	TCGTATACCAGCTCCTTGAAGGT	253	Pituitary	25	58	U12932
LHR	CTCACCTATCTCCCTGTCAAAGT	ATGGACTCATTATTCATCCCTTG	365	Testis	27	60	M81310
FSHR	GGTTCCTTATGTGTAACCTCGCC	AACTATTGGTGACTCTGGGAGCC	852	Testis	25	60	AF095642
ABP	CTATGCCTGAGACAAATCTCTGC	ATAGAAAGGACTTCCATCTTTGC	660	Testis	25	60	NM011367
AhR	GGGAGCCCAGTCTTTCCTGCTAG	ACCCTCCATCCTGGAAATTCGAA	505	Testis	27	60	M94628
Arnt	GATGCGATGATGACCAGATGTG	CAGTGAGGAAAGATGGCTTGTAGG	299	Testis	25	60	NM009709
Cyclophilin	TGGAGATGAATCTGTAGGACGAG	TAGCACATCCATGCCCTCTAGAA	382	Testis	22	60	M60456
				Adrenal	22	60	
				Pituitary	23	60	

FSH receptor (FSHR), androgen binding protein (ABP), AhR, Arnt and cyclophilin. The primer sequences and PCR product sizes are summarized in Table 1. For amplification of genes, i.e., *P450scc*, *StAR*, *GnRHR*, *LHα/FSHα*, *LHβ*, *FSHβ*, *LHR*, *FSHR*, *ABP*, *AhR*, *Arnt*, and *cyclophilin*, the respective cDNA (1 μl) was amplified in a total volume of 50 μl of reaction mixture solution that consisted of 1.25 U of TaKaRa Ex Taq™ polymerase, 1 × Ex Taq™ buffer, 0.2 μM of each dNTP mixture, and 0.2 μM of specific primers. The PCR-product sizes, optimized cycles, and annealing temperatures are summarized in Table 1. The PCR products were electrophoresed on 2% agarose gels in Tris–borate buffer (TBE) and visualized by ethidium bromide staining. Product yield was determined using Scion Images software. Data were quantified by standardizing with the PCR product of cyclophilin. The PCR products were subcloned into pGEM-T Easy vectors and sequenced by the dideoxynucleotide chain termination method using the ABI Prism Big Dye Terminator Cycle Sequencing kit.

2.4. Preparation of crude testis fraction

Mouse testes were homogenized in 0.5 ml of phosphate-buffered saline (PBS) pH 7.3, containing 1% Triton-X 100, 0.2 mM phenylmethanesulfonyl fluoride, 10 mM EDTA, 1 mg/ml leupeptin using a Teflon–glass homogenizer. The homogenate was centrifuged at 5,000 × *g* for 10 min. The supernatant was subjected to SDS–PAGE and Western blot analysis. Protein concentration was determined using BCA protein assay reagent.

2.5. SDS–PAGE and Western blot analysis

Before electrophoresis, protein was incubated in SDS sampie buffer containing 20% mercaptoethanol, and boiled for 5 min. SDS–PAGE was carried out under reducing conditions and transferred onto Immobilon-P polyvinylidene difluoride membranes. After blocking with 1% BSA, the membranes were incubated with either rabbit antiserum against P450scc or goat antiserum against HSP60 at room temperature for 2 h, followed by a 1 h incubation with either HRP-conjugated goat anti-rabbit IgG or donkey anti-goat IgG, respectively. The immunoreactive proteins were detected by an ECL Western blotting detection kit and ATTA Cool-Saver (Cat. No. AE-6955, Atto Co., Tokyo, Japan). P450scc protein content was determined using Scion Images software. Relative amounts of P450scc proteins were quantified by standardizing with the immunoreactive HSP60.

2.6. Hormone assay

The concentration of intratesticular testosterone (ITT) was determined by using an EIA kit. To measure concentrations of ITT, the frozen testis specimens were thawed and homogenized in PBS with a Teflon–glass homogenizer. The homogenate was then subject to extraction with diethyl ether, and the ether phase was dried in air. The dried lipophilic substances were resuspended with an appropriate volume of EIA buffer, and the measurements were made according to the procedure described by the manufacturer.

2.7. Statistical analysis

For statistical analysis, StatView for Windows version 5.0 (SAS Institute, Cary, NC, USA) was used. All results represented are means ± S.E. A two-tailed Student's *t*-test was used to analyze the difference in means between the means between the TCDD-treated or EB treated and vehicle groups. Data for comparison of the dose-dependent effect of TCDD using the semiquantitative RT-PCR analysis were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's PLSD test as a post-hoc test. *P*-values

<0.05 were considered to indicate a statistically significant difference.

3. Results

3.1. Effects of TCDD on levels of P450scc and testosterone in the wild-type mouse testis

TCDD was intraperitoneally administrated to 12-week-old male mice at a dose of 0, 0.8, 4, 20, or 100 μg/kg bw. After 7 days, the expression of *P450scc* in the testis was analyzed by the semiquantitative RT-PCR. The expression of *P450scc* decreased in a dose-dependent manner with a significant difference at a dose of 20 and 100 μg TCDD/kg bw (Fig. 1A). At a dose of 100 μg TCDD/kg bw in treated-mice, the expression of *P450scc* was approximately 54% of that of control mice. In the experiments that followed, we selected a dose of 100 μg TCDD/kg as a representative dose.

We next addressed the question of how TCDD affects the enzyme amounts of P450scc in the testis. The Western blot analysis with anti-P450scc antibody showed a significant decrease of P450scc in the testis ($P < 0.01$) (Fig. 1B). In these, the concentration of ITT was found to be approximately 56% of the control mice (Fig. 1C). To examine whether there was an effect of TCDD on the mobilization of cholesterol to P450scc in the testis, we used the semiquantitative RT-PCR to analyze the expression of *StAR* that plays an important role in cholesterol supply to P450scc located on the matrix side of the inner mitochondrial membrane (Stocco and Clark, 1996). No difference in the expression of *StAR* was found between the control and TCDD-treated mice (Fig. 1D).

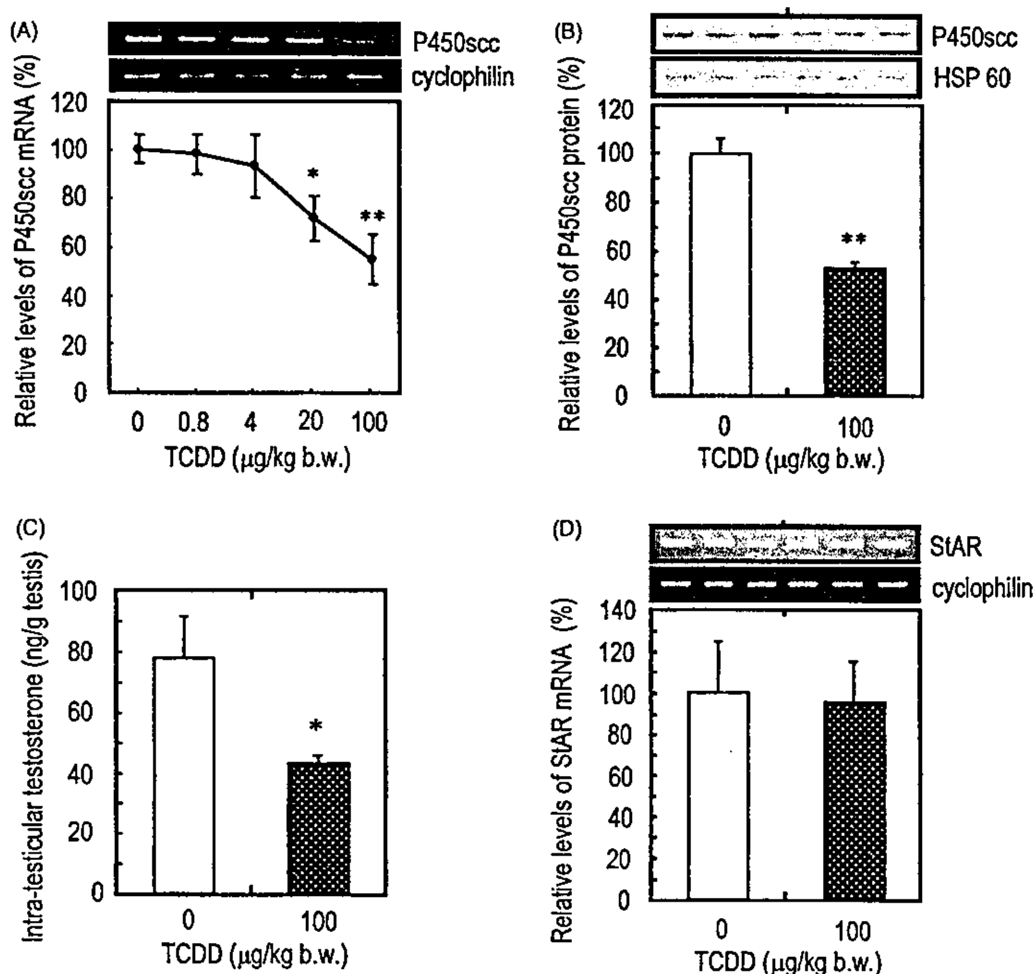

Fig. 1. Effects of TCDD on testosterone synthesis in wild-type mouse testis. (A) Dose-related effects of TCDD on P450scc mRNA analyzed by semiquantitative RT-PCR. The typical gel patterns are shown (upper panels). Histograms exhibit relative levels of those RT-PCR products to that of *cyclophilin*. (B) Effects of TCDD on P450scc protein production analyzed by Western blotting. Representative signals of three mice from each groups are shown in the upper panels of figure. Relative amounts of P450scc were determined by densitometric analysis. Results are plotted as relative levels of P450scc/HSP60 ratio. (C) Testicular testosterone concentration in TCDD-exposed adult mouse. (D) Relative levels of StAR mRNA analyzed by semiquantitative RT-PCR. Representative gels of three mice from each groups are shown (upper panels). The values were expressed as means ± S.E. for a total of five, six, four and six samples for (A)–(D), respectively. Asterisks denote a significant difference from control levels ((*)$P < 0.05$; (**)$P < 0.01$) either by ANOVA followed by Fisher's PLSD test (A), or by Student's *t*-test ((B)–(D)).