Comparative uterine gene expression analysis after dioxin and estradiol administration

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Abstract

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) adversely affects many organisms. TCDD exposure is known to be associated with abnormal development, hepatotoxicity and endocrine effects. It has also been reported to have antiestrogenic activity in addition to estrogenic activity. In order to clarify the effects of TCDD in the uterus, we evaluated the patterns of gene expression after TCDD and estradiol administration. Of the 10 000 arrayed genes, only a few were affected by both estradiol and TCDD. Although the subset of genes that responded to estrogen was also activated by TCDD, the response to TCDD was more limited than that observed in response to estradiol. Therefore, according to our analysis of gene expression patterns, TCDD had partial and weak estrogenic activity in the uterus.

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Introduction

The environmental pollutant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is an aromatic hydrocarbon that has a long biologic half-life and accumulates in animals, especially those higher in the food chain. TCDD has adverse effects on the immune system, liver, embryo, endocrine system and skin, but actual mechanisms that explain the effects are largely unknown. In the case of the reproductive tract, it has been reported that TCDD exposure is associated with an increase in endometriosis (Gibbons 1993, Rier *et al.* 1993, Bois & Eskenazi 1994, Cummings *et al.* 1996, Mayani *et al.* 1997, Rier & Foster 2002) and mammary cancer (Manz *et al.* 1991, Brown *et al.* 1998). On the other hand, other epidemiologic studies have reported that TCDD exposure can reduce mammary and uterine cancer in rats (Kociba *et al.* 1978) and humans (Bertazzi *et al.* 2001), suggesting that the effects of TCDD may be exerted through multiple mechanisms.

The effects of TCDD on the reproductive tract and, more specifically, comparisons of the effects of TCDD and estrogen have been reported previously. For example, the antiestrogenic effects of TCDD are well documented (Gallo *et al.* 1986, Gierthy *et al.* 1987, Romkes *et al.* 1987, Safe *et al.* 1991), and some molecular mechanisms of toxicity have been proposed (Krishnan *et al.* 1995, Wormke *et al.* 2003). Conversely, estrogenic effects of TCDD have also been reported (Nesaretnam *et al.* 1996, Abdelrahim *et al.* 2003).

TCDD binds to an aryl hydrocarbon receptor (AhR) that is associated with heat-shock protein 90. After the ligand binds, AhR dissociates from the heat-shock protein to form a heteromeric complex with AhR nuclear translocator (ARNT). The complex then moves into the nucleus and binds to a xenobiotic response element (XRE) to activate the transcription of the TCDD response gene. The ligand-dependent behavior of AhR is similar to the estrogen receptor (ER) to some extent. When estrogen binds to the ER, the ER binds to the

estrogen-response element (ERE) to activate the transcription of estrogen-response genes. Two models are proposed to explain the estrogenic effect of TCDD. One is direct binding of TCDD to ER (Nesaretnam *et al.* 1996) and the other is ligand-dependent protein–protein interaction of AhR and ER, which has been demonstrated with 3-methylcholanthrene (Ohtake *et al.* 2003).

In the present study, we used DNA microarray analysis to investigate the estrogenic activity of TCDD. Accordingly, we determined the effects of TCDD and estrogen on gene expression and compared their gene expression patterns. By this approach, we estimated the estrogenic effects of TCDD.

Materials and methods

Animals

Female C57/BL6/J mice were purchased from SLC, Inc. (Tokyo, Japan), housed under a 12-h light:dark cycle and given free access to food and water. Mice were ovariectomized at 8 weeks of age and, 2 weeks later, either sesame oil vehicle (Nakarai Tesque, Kyoto, Japan) or test material was administered to the animals. Whole uteri ($n=4$) were collected 6 h after dosing. The doses of 17 β -estradiol (E2) (Sigma, Sigma-Aldrich Japan, Tokyo, Japan) were 0, 0.05, 0.5, 5.0 and 50 $\mu\text{g}/\text{kg}$ body weight (b.w.). The doses of TCDD (Cambridge Isotope Laboratory, Andover, MA, USA) were 0, 1.0 and 10 $\mu\text{g}/\text{kg}$ b.w. Our institutional animal care committee approved all animal experiments.

Immunohistochemistry

Uterine tissue sections (4 μm) were prepared and incubated with anti-ER, anti-AhR or anti-ARNT antibodies. Staining was performed with a Histofine kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. Briefly, deparaffinized and rehydrated tissue sections were boiled twice in citrate buffer (10 mM citrate, pH 6.0) for 5 min and pretreated with 3% hydrogen peroxide for 10 min. The anti-ER (H-184), anti-AhR (M-20) and anti-ARNT (C-19) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were incubated with the tissue sections at 4 °C overnight. For immunodetection, the sections were incubated with

a second antibody conjugated with peroxidase for 10 min and developed with 3,3'-diaminobenzidine.

DNA microarray analysis

Total uterine RNA was extracted with TRIzol (Invitrogen, Tokyo, Japan) and purified with the RNeasy minikit (Qiagen, Tokyo, Japan). Total RNA quality was examined with a Bioanalyzer 2100 (Agilent Japan, Tokyo, Japan). Purified RNA was processed according to the manufacturer's protocol to prepare the labeled cRNAs, which were hybridized to the mouse genome U74 array (Affymetrix Japan, Tokyo, Japan). Hybridization, washing and scanning were performed according to the manufacturer's protocol.

Data analysis

Scanned data were analyzed with GeneChip Suit Analysis Software ver.5.0 (Affymetrix Japan, Tokyo, Japan) to obtain the average intensity of each cell corresponding to each oligonucleotide probe. The averaged fluorescence intensity (2500) of each probe was further analyzed by dChip, a model-based expression-analysis program (Li & Wong 2001), and expression levels were estimated. The PM-only model was used for the analysis, and the estimated values were transferred to the GeneSpring software program (Silicon Genetics, Redwood City, CA, USA) and analyzed. To calculate changes in expression, genes for which average expression levels were more than 1000 fluorescence intensity units under at least one experimental condition were selected, and the average expression values of the treated samples were divided by those from control samples.

Quantitative real-time PCR

Total RNA was purified as described above. cDNA was synthesized from purified total RNA with Superscript II RT(-) (Invitrogen, Tokyo, Japan), and random primers at 42 °C for 60 min. PCR reactions were performed in the Prism 7000 sequence detector (Applied Biosystems Japan, Tokyo, Japan) with SYBR-Green PCR core reagents (Applied Biosystems Japan, Tokyo, Japan) in the presence of appropriate primers, according to the manufacturer's instructions. The primers

Figure 1 Immunolocalization of DNA-binding transcription factors. Tissue distributions of ER, AhR and ARNT in uterus were examined by immunohistochemistry. Rather intense signals were detected in epithelial tissues. A clear difference in staining level between the myometrium and stroma was not observed for ER, AhR and ARNT. Magnification, $\times 400$ ER (A), AhR (B) and ARNT (C).

were chosen to amplify short PCR products of less than 100 base pairs, and their sequences are as follows:

- NM_007636 (chaperonin subunit 2; *Cct2*)
CGCTGCTGGCGTCATG, TGACAAGAG
CGAGGCGCT
- NM_019768 (mortality factor 4 like 2;
Morf4l 2) AGCTGGTTTCGCAGCCAAA,
CAGAGCGACCTCCCCATCT
- NM_009455 (ubiquitin-conjugating enzyme
E2E 1; *Ube2e1*) GCTATGTCGGATGAC
GATTCG, TCTCGGTCTGCTGGTTGGA
- NM_007479 (ADP-ribosylation factor 4; *Arf4*)
CCTCCTTTTGACCGTCAAGTG, AACAGGCGCGAGAAGAGAGA
- NM_011045 (proliferating cell nuclear anti-
gen; *Pcna*) GCGCAGAGGGTTGGTAG
TTG, CCCGATTACGATGCAGAA
- NM_009505 (vascular endothelial growth
factor A; *Vegfa*) ACATCTTCAAGCCGTCC
TGTGT, CTCCAGGGCTTCATCGTTACA
- NM_012053 (ribosome L8, *Rpl8*, used as
control) ACAGAGCCGTTGTTGGTGT
TG, CAGCAGTTCCTCTTTGCCTTGT.

Each PCR amplification was performed in triplicate in the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). Model 7000 software was used to construct amplification plots from extension-phase fluorescent emission data collected during PCR amplification. Threshold (C_T) values were calculated by determining the point at which fluorescence exceeds a threshold limit.

Gene expression levels were normalized to the expression levels of L8 mRNA (U67771), and changes in concentration were calculated. Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. Quantification of mRNAs was repeated three times with independent mice, and average levels of change were calculated.

Results

Expression of AhR in uterus

To examine the effects of TCDD and estradiol on uterine gene expression, we first examined the

Figure 2 Gene expression changes induced by TCDD. Uterine gene expression after TCDD treatment was examined by DNA microarray. (A) Scatter plot of gene expression levels after TCDD exposure (y-axis) versus control gene expression levels (indicated by oil, x-axis). Gene expression levels correspond to the fluorescence intensity of the scanned DNA microarray. (B) Changes in gene expression levels upon treatment with 1 µg/kg (x-axis) or 10 µg/kg (y-axis) TCDD. The plot corresponding to cytochrome P450 1A1 gene is indicated as 'CYP1A1'.

Table 1 Changes in TCDD activated genes in the uterus. Changes in gene expression levels based on oil treatment were calculated for both doses (1 and 10 µg/kg). Only the genes that were activated more than two-fold by 10 µg/kg of TCDD are indicated. Genes that were also activated more than 1.5-fold by estradiol (see also Figure 4) are indicated in bold.

Genbank Accession	TCDD (µg/kg)			Definition
	0	1	10	
K02588	1.0	2.3	10.3	cytochrome; cytochrome P450
X13060	1.0	10.8	7.8	LINE repetitive sequence; repetitive sequence; serum albumin
Y14660	1.0	7.4	5.8	<i>fabp1</i> gene; fatty acid binding protein; L-FABP
M81445	1.0	2.1	4.7	connexin; transmembrane protein
M60523	1.0	1.1	3.8	growth factor-inducible protein; helix-loop-helix protein
M16360	1.0	3.0	2.8	major urinary protein; tissue specific gene
D16333	1.0	1.9	2.8	coproporphyrinogen oxidase; coproporphyrinogenase
M17818	1.0	3.1	2.6	urinary protein
M95604	1.0	1.1	2.6	mesoderm; zinc-finger protein
M69260	1.0	2.6	2.5	lipocortin 1
X00945	1.0	3.1	2.5	protease inhibitor
X99347	1.0	1.9	2.5	<i>LBP</i> gene; LPS-binding protein
M31885	1.0	0.9	2.5	helix-loop-helix DNA binding protein regulator
M95200	1.0	0.6	2.4	vegfa gene; vascular endothelial growth factor
D10627	1.0	1.6	2.4	zinc finger protein
AB009275	1.0	2.3	2.4	matrin3
AB015140	1.0	0.9	2.3	AhR repressor
X66091	1.0	2.0	2.3	alternative splicing factor; <i>ASF</i> gene; RNA-binding protein
X60452	1.0	2.4	2.3	cytochrome P450; cytochrome P450 IIIA
X91656	1.0	1.8	2.3	splicing factor; <i>SRp20</i> gene
M12347	1.0	2.4	2.3	actin; alpha-actin
X92665	1.0	2.1	2.2	<i>UbcM3</i> gene; ubiquitin-conjugating enzyme
X17320	1.0	2.3	2.1	antigen; <i>pcp4</i> gene
AB023564	1.0	2.2	2.1	type I peroxiredoxin
X99963	1.0	1.5	2.0	<i>rhoB</i> gene
X57800	1.0	2.1	2.0	Pcna gene; proliferating cell nuclear antigen
AB022156	1.0	1.6	2.0	chaperonin containing TCP-1 beta subunit
D87901	1.0	1.8	2.0	Arf4 gene; ADP-ribosylation factor

spatial expression pattern of AhR and compared it with the expression pattern of ER and ARNT. Immunohistochemistry localized AhR and ARNT within the uterine tissues and showed that the expression pattern of AhR was very similar to that of the transcription factors ER and ARNT (Fig. 1). All three proteins were expressed to a greater degree in luminal and glandular epithelium than in other tissues, and clear differences in expression levels could not be observed between stroma and myometrium.

Effect of TCDD on uterine gene expression

To profile uterine genes affected by TCDD, mice were ovariectomized to eliminate the effect of endogenous estrogen; 2 weeks later, they were treated with 10 µg/kg TCDD. Six hours after

treatment, uteri were removed and RNA was isolated. We examined the expression of about 10 000 genes by DNA microarray analysis. As shown in Fig. 2A, the scatter plot of gene expressions treated by TCDD versus control showed a diagonal line, indicating that most gene expression levels did not change to a large extent, and that there was little bias during data acquisition and analysis. As a result, it was found that 28 annotated genes were activated and 20 annotated genes were repressed more than twofold after filtering the data (see Materials and methods). Lists of the activated and repressed genes are provided in Tables 1 and 2 respectively.

To determine whether the genes responded to TCDD in a dose-dependent manner, we treated additional mice with 1 µg/kg TCDD, monitored changes in their gene expression and compared the

Table 2 Changes in TCDD repressed genes in the uterus. Changes in gene expression levels based on oil treatment were calculated for both doses (1 µg/kg and 10 µg/kg). Only the genes that were repressed more than two-fold are indicated. Genes related to immunoglobulin are marked with asterisks.

Genbank Accession	TCDD (µg/kg)			Definition
	0	1	10	
L43568	1.0	0.5	0.5	*antigen, B-cell receptor
AB001607	1.0	0.6	0.5	prostacyclin synthase
X04673	1.0	0.5	0.5	adipsin; inverted repeat; serine protease; tandem repeat
M20497	1.0	0.6	0.4	adipose fatty acid-binding protein; binding protein
L04961	1.0	0.2	0.4	inactive X-specific transcript
X03505	1.0	0.3	0.4	serum amyloid A protein; serum protein
X88903	1.0	0.4	0.4	*light chain; variable region
AJ006474	1.0	0.6	0.4	ca3 gene; carbonic anhydrase; carbonic anhydrase III; promoter region
D26090	1.0	0.4	0.3	mCDC46; CDC46; mCDC46 protein; DNA replication
L14553	1.0	0.3	0.3	*immunoglobulin light chain; monoclonal antibody; rearranged gene; variable region
Z70661	1.0	0.4	0.3	*Ig kappa light chain; Ig light chain; immunoglobulin; joining region; variable region
M12481	1.0	1.6	0.3	beta-actin
X67210	1.0	0.2	0.3	*constant region; immunoglobulin; immunoglobulin heavy chain; variable region
X00652	1.0	0.3	0.3	*Ig kappa light chain; Ig light chain; immunoglobulin; joining region; variable region
X16678	1.0	0.3	0.3	*Ig kappa light chain; variable region; Vk gene
X02468	1.0	0.3	0.2	*germ line; Ig heavy chain; immunoglobulin; variable region; variable region subgroup VH-II
M19681	1.0	0.2	0.2	JE protein; cytokine
M90766	1.0	0.2	0.1	*immunoglobulin mu-chain; joining chain; polymeric IgM, IgA
M80423	1.0	0.2	0.1	*C-region; immunoglobulin light chain; immunoglobulin mu-chain
J00475	1.0	0.2	0.1	*C-region; alternative splicing; germline; immunoglobulin heavy chain; immunoglobulin-alpha

results to those from the 10 µg/kg dose (Fig. 2B). The cytochrome p450 1A1 gene exhibited a dose-dependent activation pattern, as indicated by its location near the y-axis on a plot of gene expression levels (designated CYP1A1 in Fig. 2B). On the other hand, the expression levels of many other genes plotted onto a nearly diagonal line indicated that these genes were not activated in a dose-dependent fashion at 1–10 µg/kg. Many of the repressed genes generally exhibit immune system function, such as immunoglobulin chains and B-cell receptor antigen.

Comparison of TCDD gene-activation pattern with estrogen effects

To determine whether the effects of TCDD on uterine gene expression are similar to estradiol, we analyzed and compared the gene expression patterns after treatment with both agents. The genes that were activated or repressed more than twofold by either TCDD or estradiol at 6 h were selected for further analysis. The 575 selected

genes, which included *EST* genes, were analyzed by hierarchic clustering (Fig. 3). According to the dendrogram, this activation pattern was divided into six primary clusters, as indicated in Table 3 (Fig. 3A–F, dendrogram is not shown).

Genes in cluster A were activated by both TCDD and estradiol, although the gene expression changes induced by TCDD were much smaller than those induced by estradiol. Average changes in response to TCDD were comparable to 0.5 µg/kg estradiol. Many genes clustered in B were activated only by estradiol, and not by TCDD. From this clustering analysis, the number of genes affected by both TCDD and estradiol was rather limited, and only a portion of estrogen-activated genes were weakly activated by TCDD.

DNA microarray analysis suggested that six of the TCDD-induced genes were activated more than twofold by estradiol. Three genes were confirmed to be activated more than 1.5-fold by both TCDD and estradiol by quantitative PCR, although the changes were not consistent with those observed on the DNA microarray (Fig. 4).

Figure 3 Distinct gene expression patterns after TCDD and estradiol (E2) administration. The genes that were activated more than twofold by either TCDD or estradiol were selected, and their expression patterns were analyzed by hierarchic clustering. According to the dendrogram (not shown), the expression patterns were divided into six classes (A–F). The average changes for each class are indicated on the right. The numbers indicate the number of genes classified into each class. Dose increments are indicated by triangles, which correspond to 0, 1 and 10 µg/kg for TCDD, and 0.05, 0.5, 5 and 50 µg/kg for estradiol (from left to right).

Table 3 Gene activation pattern of TCDD or E2 induced genes. Based on the result of hierarchical clustering, six clusters were found (Fig. 3). Averaged gene expression pattern in each cluster is indicated

Cluster	TCDD	Estradiol
A	Slightly activated	Activated
B	Not activated	Activated
C	Mainly activated	Not activated
D	Weakly activated	Repressed
E	Not repressed	Repressed
F	Repressed	Not affected

These genes included vascular endothelial growth factor A (*Vegfa*, NM_009505), proliferating cell nuclear antigen (*Pcna*, NM_011045) and ADP-ribosylation factor 4 (*Arf4*, NM_007479) (Kim *et al.* 2003) (indicated in boldface in Table 1).

Temporal changes in TCDD-induced genes in response to estrogen

Since the number of genes activated by both TCDD and estradiol was rather small, we selected a gene set that was induced by TCDD at 6 h and examined its temporal activation pattern after estradiol treatment. If the kinetics of AhR are much slower than those of ER, gene activation through AhR may be slower than that through ER. In this case, even if the set of activated genes is similar, temporal expression patterns may differ, and genes activated by AhR at 6 h may be similar to those activated by ER at a much earlier time point. Although several TCDD-responsive genes were activated at 1–2 h by estradiol (Fig. 5A), some other genes were activated much later (Fig. 5B), and no time point was identified at which the majority of TCDD-responsive genes were activated simultaneously. This result indicates that the E2-affected genes and the TCDD-affected genes did not overlap temporally.

Discussion

TCDD-affected genes

To examine the estrogenic effects of TCDD, we used DNA microarray analysis to evaluate uterine gene expression after treatment with TCDD or estrogen. The number of TCDD-affected genes

Figure 4 Confirmation of gene activation by either TCDD or estradiol (E2). According to the DNA microarray analysis, genes commonly activated by TCDD and estradiol were selected, and their expression was confirmed by quantitative PCR. Expression levels were normalized to the expression of ribosomal L8 protein gene, and the calculated changes are indicated.

was rather restricted compared with estrogen-affected genes (Watanabe *et al.* 2002, 2003). Several hundred genes were affected by estrogen treatment, whereas a high dose of TCDD affected only 120 genes, of which 48 were known genes. CYP1A1 was induced in a TCDD dose-dependent manner (Sadek & Allen-Hoffmann 1994), and AhR repressor (AhRR) (Mimura *et al.* 1999) was also activated, indicating that the doses in the present study were within the AhR-effective range. Previous studies have demonstrated that cytochrome P450 1A1 gene and AhRR are activated via AhR in various tissues and cultured cells (Mimura *et al.* 1999, Whitlock 1999). These genes are also activated in the uterus. Although several connexin genes are downregulated in the liver in response to TCDD, in our study, connexin 26 gene (M81445) was activated in the mouse uterus.

In addition to the genes showing dose-dependent activation, such as CYP1A1, many TCDD-affected genes were not activated dose-dependently. The genes that were equally activated at either dose might reach an induction plateau by 1 µg/kg TCDD; thus, the expression levels remain constant even at the higher dose. Likewise, other transcription factors may contribute to gene activation. For example, Cyp3A gene is activated by both SXR (Rosenfeld *et al.* 2003) and TCDD.

Of the genes repressed by TCDD, those related to immunoglobulin were not affected by estradiol. Several studies have described the effects of TCDD on the immune system (Baccarelli *et al.* 2002) and suppression of NF-κB activity (Tian *et al.* 1999). Since NF-κB affects immunoglobulin gene expression, repression of the genes related to immunoglobulin may be related to the adverse effects of

Figure 5 Temporal changes in the selected genes after estrogen treatment. The genes that were induced more than twofold by TCDD treatment were selected, and their expression levels after estrogen treatment were examined. The genes that showed greater changes in response to estradiol at 1–2 h are indicated in panel A, and the genes that showed greater changes in response to estradiol at 6–48 h are indicated in panel B. The remaining genes were not activated more than twofold by estradiol at any time point.

TCDD on the immune system. Alternatively, the number of immune cells that migrate to the uterus may be reduced after TCDD treatment.

TCDD and E2-induced genes

Proliferating cell nuclear antigen (*Pcna*) and vascular endothelial growth factor (*Vegf*) were commonly activated by TCDD and E2. Theoretically, if *Vegf* and *Pcna* were activated by the estrogenic activity of TCDD, other genes that were efficiently activated by estrogen should be activated by TCDD. Since not all genes activated by estradiol were activated by TCDD, this suggests that TCDD has only partial estrogenic activity. In this context, a clearer understanding of the *Vegf* gene activation mechanism in response to either TCDD or estradiol may provide important information on TCDD's genetic effects.

Our DNA microarray analysis indicated that only a portion of the estrogen-activated genes (cluster A in Fig. 3) was activated by TCDD, and a larger number of genes were not activated (cluster B in Fig. 3). Two models have been proposed to explain the estrogenic effects of TCDD. The first

focuses on the induction of ER by TCDD (Nesaretnam 1996), and the second model, based on a study of 3-methylcholanthrene, suggests a direct interaction between AhR and ER (Ohtake 2003). Although our data do not directly support these models, it can be concluded that the estrogenic effects of TCDD *in vivo* are very limited and that TCDD mimics estrogen only under very restricted conditions in the uterus. Because AhR and ER exhibit very similar distribution throughout the uterus (Fig. 1), the partial estrogenic activity of TCDD may not be related to the tissue-specific distribution of these receptors in the uterus.

In summary, we examined the effects of TCDD on uterine gene expression and demonstrated only weak estrogenic activity. A better understanding of the genes that are activated by both TCDD and estrogen will contribute to a more complete understanding of TCDD's effects on the uterus.

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