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Toxicology 204 (2004) 51–60
TOXICOLOGY
www.elsevier.com/locate/toxicol

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

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Received 12 February 2004; accepted 3 June 2004
Available online 28 July 2004

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.

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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doi:10.1016/j.tox.2004.06.024

nated biphenyls (PCBs) are widespread and persistent environmental pollutants. Among them is the most toxic compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is known to exert diverse biological effects including immunotoxicity, reproductive deficits, neurobehavioral toxicity, and carcinogenicity (Birnbaum and Tuomisto, 2000; Dragan and Schrenk, 2000; Tohyama, 2002). A major part of the TCDD toxicities can be mediated by a ligand-dependent transcription factor, the arylhydrocarbon receptor (AhR). Upon entering cells, TCDD binds AhR in the cytoplasm and causes a translocation of the liganded-receptor to the nucleus, where the AhR heterodimerizes with another transcription factor, the arylhydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT heterodimer induces expression of various genes by binding xenobiotic responsive elements (XREs) in the promoter regions (Schmidt and Bradfield, 1996; Wilson and Safe, 1998; Mimura and Fujii-Kuriyama, 2003) or modifies cellular functions through protein–protein interactions (Carlson and Perdew, 2002; Swanson, 2002). In addition to TCDD, six congeners of PCDDs, 10 of PCDFs, and 12 of coplanar PCBs are known to bind to AhR and elicit AhR-mediated biological and toxic responses, although the magnitude of the effects widely varies among the compounds (Van den Berg et al., 1998). These compounds are called dioxins (for PCDDs and PCDFs), or dioxin-like PCBs.

The toxic equivalency factor (TEF) is commonly used in the assessment of the health risk of exposure to dioxins and dioxin-like compounds that occur as mixtures in the environment (Birnbaum and DeVito, 1995; Van den Berg et al., 1998). The TEF value for each dioxin-related compound is the order of magnitude estimate of the AhR-dependent toxicity relative to TCDD, which is derived using scientific judgment after considering all available relative potency (REP) data (Van den Berg et al., 1998). The underlying REP data practically shows great variability and heterogeneity for individual congeners, especially for PCBs (Finley et al., 2003). The toxic effects of dioxin-related compounds are known to be organ-, cell-, and species-specific, and their sensitivities depend on the endpoints in various organs or cells. These factors, such as organ- or cell-type, species, and endpoints, are suggested to be significant contributors to the variability of REPs (Finley et al., 2003). Differences in pharmacokinetics among the compounds can also affect the variety of REPs. Some compounds are known to be more effectively sequestered, possibly by binding with CYP1A2, in the liver than TCDD, which may alter the REPs of these compounds (DeVito et al., 1998).

The TEFs were primarily derived from in vivo toxicity REP data, which were given more weight than in vitro and/or quantitative structure-activity relationship data (Van den Berg et al., 1998). Among various toxic endpoints, the immune system is recognized as one of the most vulnerable organs to TCDD toxicity. In mice, TCDD induces thymus involution and suppresses both humoral and cellular immunity (reviewed in Holsapple et al., 1991; Vos et al., 1997/1998; Kerkvliet, 2002). In our recent researches, studying the effects of TCDD exposure on the humoral immune reactions in mice immunized with OVA (Nohara et al., 2002; Fujimaki et al., 2002; Ito et al., 2002; Inouye et al., 2003), we found that the production of Th2 type cytokines, especially IL-5, by splenocytes are greatly and sensitively suppressed by TCDD exposure (Ito et al., 2002). The deficit in T cell functions was suggested to be involved in the suppression of antibody production by TCDD. A study conducted in our laboratory further showed that the inhibition of IL-5 production reflected TCDD toxicity at a lower dose than the endpoints, such as suppressed antibody production or thymus atrophy (Inouye et al., unpublished data). These endpoints sensitively detect the adverse health effects that would be useful in the risk assessment of dioxins (WHO, 2000).

In the present study, we re-evaluated REPs for immunotoxicity of toxic members of PCBs, PCB126 (TEF 0.1) and PCB169 (TEF 0.01), using indexes including IL-5 production by splenocytes. Since the REP data for PCB126 have been greatly accumulated and the variability is not as large as those of PCB169, this congener was chosen for a reference purpose in addition to TCDD. On the other hand, the REP data for PCB169 are largely fluctuated among studies (Finley et al., 2003). Some even found that the REP values were much higher than 0.01 for PCB169 (Harper et al., 1995; Mayura et al., 1993). In order to reason the mechanism of determining the REP of PCB169 in immunotoxicity, we also measured the tissue concentration of the chemicals and CYP1A1 induction, the established sensitive marker for activated AhR function, in the spleens and livers in mice dosed with PCB169 and TCDD.

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,

Foster City, CA), 0.25 U of UDP-N-glycosidase, and 100 nM CYP1A1 primer set (5′-CCTCTTTGGAGCTGGGTTTG-3′ and 5′-GCTGTGGGGGATGGTGAA-3′). The thermal cycling comprised an initial step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min, and an amplifying step with 50 cycles at 95 °C for 15 s and at 63 °C for 1 min. A melting curve was examined to make sure that only the amplicon but not primer dimers accounted for SYBR Green-bound fluorescence. Assays were performed in duplicate with an ABI Prism 7000 Sequence Detector (Applied Biosystems). All data were normalized to an internal standard glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The primers for G3PDH were 5′-CTGCACCACCAACTGCTT-3′ and 5′-CCAGTGAGCTTCCCGTTC-3′.

2.8. Tissue concentration of TCDD and PCB169

The livers and spleens were removed from the mice on day 4 after administration and immunization. Tissue samples of individual groups were pooled and digested in 2 M of KOH for 12 h. A portion of the digested material was weighted and spiked with ^{13}C-PCDDs/PCDFs or ^{13}C-PCBs (Wellington, Ontario, Canada) as an internal standard. An extraction of chemicals and their cleanup were carried out, and the GC/MS analysis was conducted according to the method described by Nohara et al. (2000).

2.9. Statistical analysis

StatView statistical software (StatView-J 5.0, SAS Institute, Cary, NC) was used in our statistical analysis. Differences in means among the groups were analyzed by one-way ANOVA, followed by Dunnett's test as a post hoc comparison. The values are presented as the mean ± the standard error (S.E.), and the P values less than 0.05 were considered statistically significant.

3. Results

3.1. Relative potency of PCB126 on immune organs and functions

Recently, we conducted time course studies on the effects of TCDD on the IgM and IgG1 amount in the plasma, and IL-5 production by splenocytes in mice immunized with OVA (Ito et al., 2002; Inouye et al., 2003). In the present study, we compared the effects of PCB126 and TCDD on the same indexes at individual time points when a maximum inhibitory effect of TCDD was observed for each target. Mice were dosed with 20 μg TCDD/kg or 200 μg PCB126/kg based on the established TEF value (0.1) for PCB126, and subsequently immunized with OVA. These compound doses did not affect the body weights (data not shown). As shown in Fig. 1, TCDD (20 μg/kg) and PCB126 (200 μg/kg) induced the same extent of thymus atrophy on day 4 after animal treatments. Liver weight increase, a well-known sign of TCDD exposure, was also observed by TCDD and PCB126 to the same extent. Spleen weight was not changed by TCDD or PCB126 (data not shown). Characteristic features of changes in thymocytes by TCDD exposure are reductions in the total cell number and the $CD4^+CD8^+$ double positive (DP) cell percentage, and an increase in the CD8 single positive cell percentage. As summarized in Table 1, all features were observed by exposure of TCDD or PCB126. The chemicals did not affect the lymphocyte populations in the spleens (data not shown).

IgM production measured on day 10 after immunization was also inhibited by these two compounds to the same extent (Fig. 2). However, IgG1 production was suppressed by 20 μg TCDD/kg, but not by 200 μg PCB126/kg (Fig. 2). These results were confirmed in another independent experiment (data not shown). Fig. 3 shows the IL-5 production by splenocytes from mice dosed with TCDD or PCB126. Both TCDD (20 μg/kg) and PCB126 (200 μg/kg) induced a significant suppression in IL-5 production.

In summary, exposure to 20 μg TCDD/kg and 200 μg PCB126/kg had qualitatively, as well as quantitatively, similar effects on immune organs and the liver, except for IgG1 production being inhibited by TCDD but not by PCB126.

3.2. Relative potency of PCB169 on immune organs and functions

We also examined the potency of PCB169 (TEF 0.01) relative to TCDD. In comparison with 20 μg TCDD/kg, mice were dosed with 2000 or 5000 μg PCB169/kg, an amount equal to or larger than 20 μg TCDD/kg as the potency based on the TEF. These

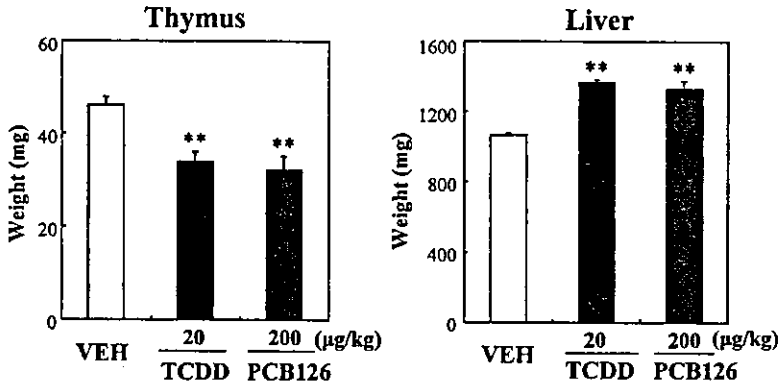

Fig. 1. Thymus and liver weight in the mice exposed to TCDD or PCB126. Mice were given corn oil (vehicle, VEH), TCDD or PCB126 and subsequently immunized with OVA/alum. The thymus and liver weights were measured 4 days after the treatments. Results are presented as mean ± S.E. ($n = 5$). Asterisks (**) indicate a statistically significant difference from the corresponding control with $P < 0.01$.

Table 1
Thymocyte number and subpopulation in mice exposed to TCDD or PCB126

Group	Thymocytes ($\times 10^7$)	Subpopulations (%)			
		DN[a]	DP[a]	CD4SP[a]	CD8SP[a]
Vehicle	7.6 ± 0.9	4.5 ± 0.2	79.1 ± 1.2	10.7 ± 0.8	5.8 ± 0.3
TCDD (20)	2.1 ± 0.4**	6.2 ± 0.3**	57.1 ± 3.0**	23.0 ± 2.1**	13.7 ± 0. 8**
PCB126 (200)	3.4 ± 0.7**	5.9 ± 0.1**	68.5 ± 1.6**	15.2 ± 1.1	10.4 ± 0.6**

Mice were given corn oil (vehicle), TCDD or PCB126 (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. Statistically significant difference from the vehicle-treated mice with *$P < 0.05$. **$P < 0.01$.

[a] DN, double negative cells; DP, double positive cells; SP, single positive cells.

compound doses did not affect the body weights (data not shown).

Although liver weight was significantly increased by TCDD (20 μg/kg) and PCB169 (2000 or 5000 μg/kg), thymus involution was not significantly induced by 2000 μg or even 5000 μg PCB169/kg in contrast to TCDD (Fig. 4). As shown in Table 2, 2000 μg PCB169/kg did not lead to significant reduction in the

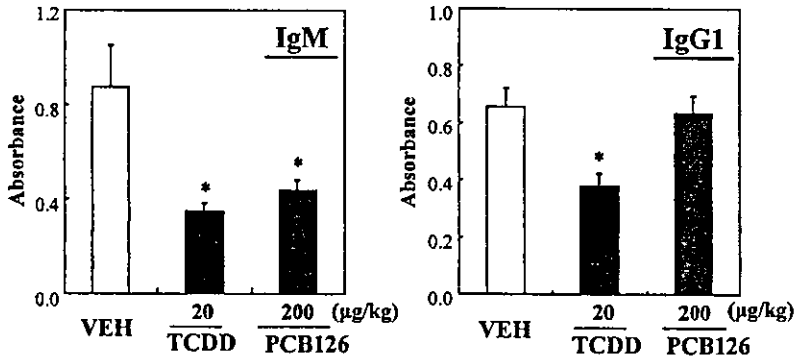

Fig. 2. Effect of TCDD- or PCB126-exposure on the OVA-specific antibody production. Mice were treated as described in the legend for Fig. 1. Ten days after the treatments, OVA-specific IgM and IgG1 antibodies in the plasma were measured by ELISA. Results are presented as mean ± S.E. ($n = 5$). *$P < 0.05$.

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.

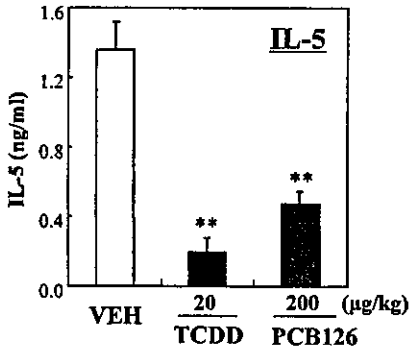

Fig. 3. Effect of TCDD- or PCB126-exposure on the interleukin-5 (IL-5) production by the splenocytes re-stimulated with OVA ex vivo. Mice were treated as described in the legend for Fig. 1. Four days after the treatments, the splenocytes were prepared and equal numbers of cells were cultured with OVA 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

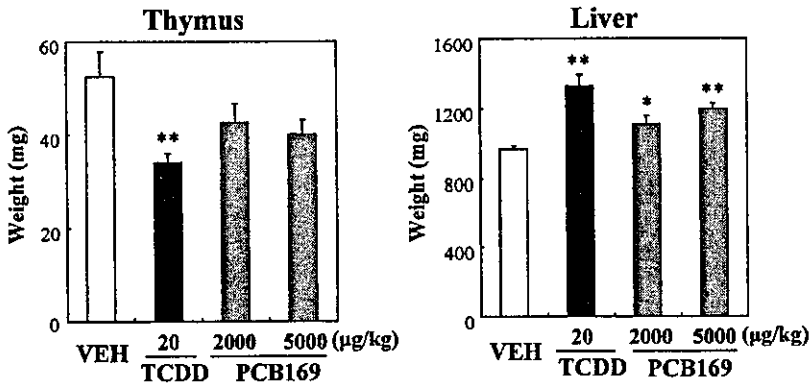

Fig. 4. Thymus and liver weight in the mice exposed to TCDD or PCB169. Mice were given corn oil (vehicle, VEH), TCDD or PCB169 and subsequently immunized with OVA/alum. The thymus and liver weights were measured 4 days after the treatments. Results are presented as means ± S.E. ($n = 5$). $^{*}P < 0.05$,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

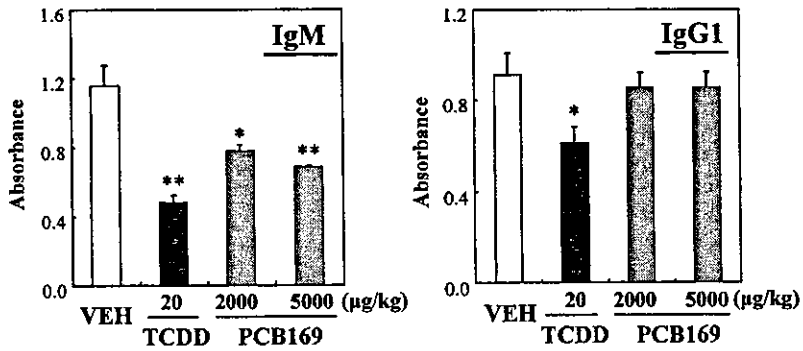

Fig. 5. Effect of TCDD- or PCB169-exposure on the OVA-specific antibody production. Mice were treated as described in the legend for Fig. 4. Ten days after the treatments, OVA-specific IgM and IgG1 antibodies in the plasma were measured by ELISA. Results are presented as mean ± S.E. ($n = 5$). $^{*}P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

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

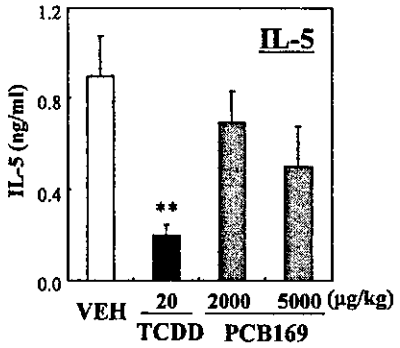

Fig. 6. Effect of TCDD- or PCB169-exposure on the interleukin-5 (IL-5) production by the splenocytes re-stimulated with OVA ex vivo. Mice were treated as described in the legend for Fig. 4. Four days after the treatments, the splenocytes were prepared and equal numbers of cells were cultured with OVA 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.

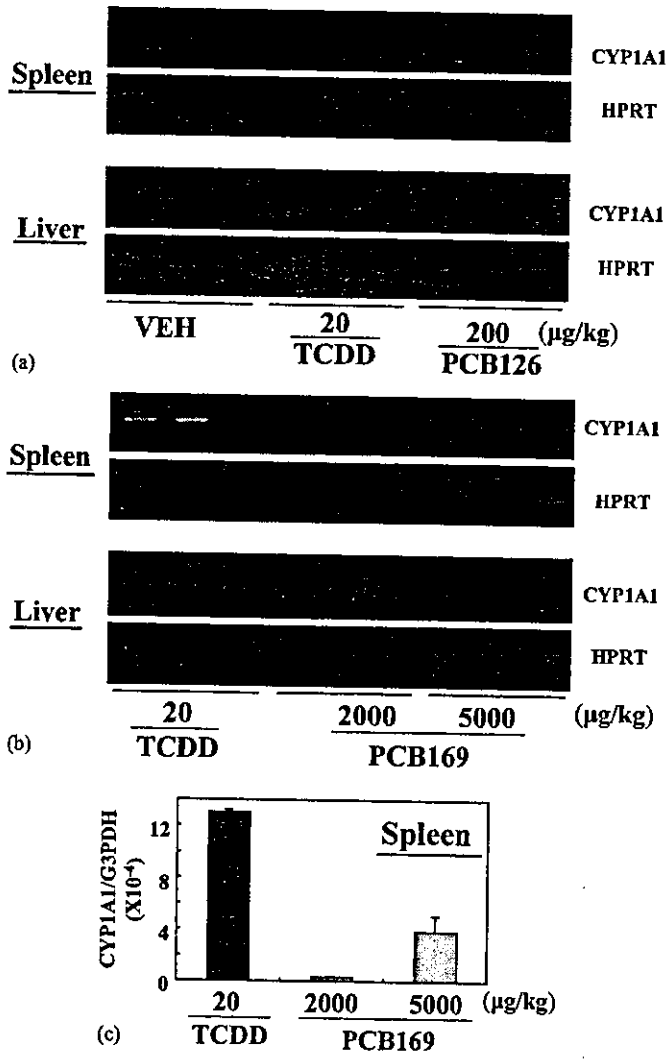

Fig. 7. Expression of CYP1A1 mRNA in the spleens and livers of TCDD- or PCB-exposed mice. Mice were given either corn oil (vehicle, VEH), TCDD, PCB126 or PCB169 and subsequently immunized with OVA/alum. Four days after the treatments, expression of CYP1A1 mRNA in the livers or spleens was measured by 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. ($n = 5$).

Acknowledgements

The authors wish to thank Dr. J. Yonemoto, Dr. H. Fuijmaki (NIES) and Dr. M. Kubo (Science University of Tokyo) for their helpful discussions, M. Matsumoto for her technical assistance and K. Nakazawa for her secretarial assistance. This work was supported in part by grants from CREST, JST, the Ministry of Health, Labor and Welfare (C.T.), and the Ministiry of the Environment.

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Molecular and Cellular Endocrinology 221 (2004) 87–96

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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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Received 27 November 2003; accepted 24 February 2004

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.

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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doi:10.1016/j.mce.2004.02.005

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 sample 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).

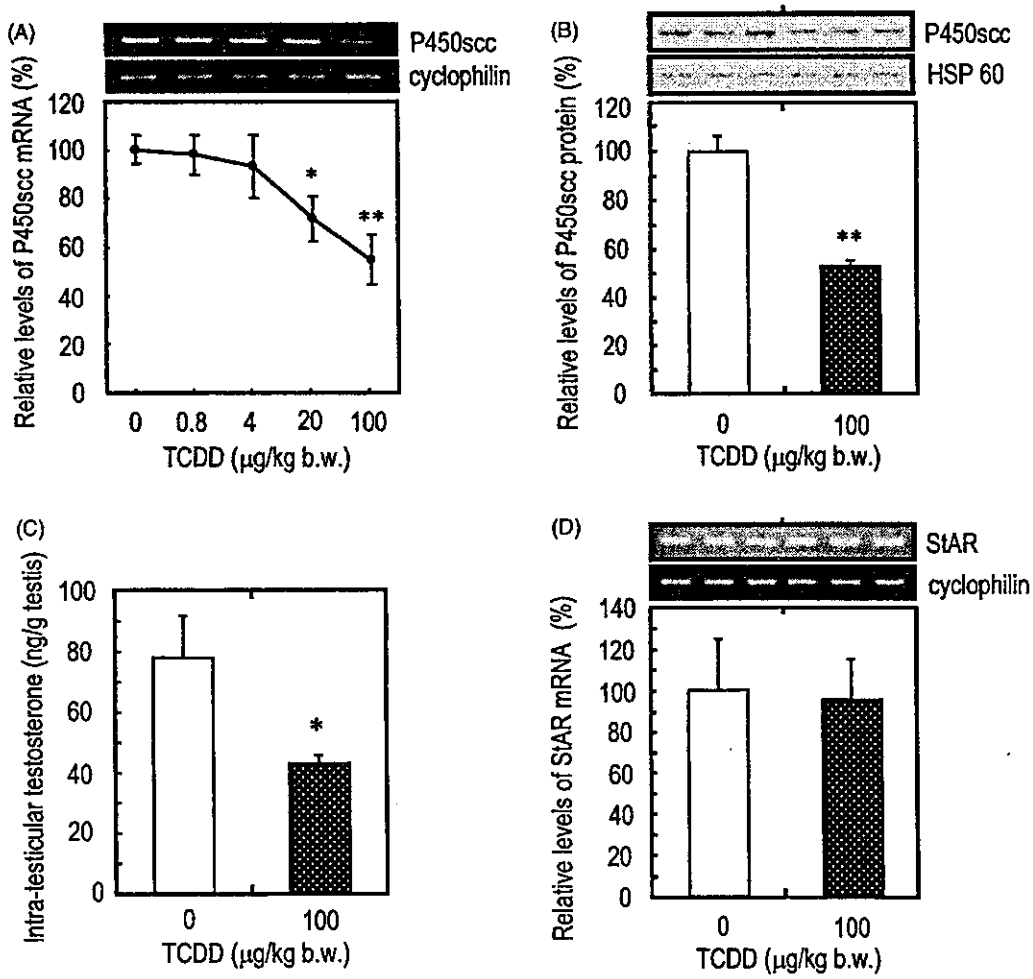

Fig. 1. Effects of TCDD on testosterone synthesis in wild-type mouse testis. (A) Dose-related effects of TCDD on P450scc mRNA analyzed by semiquantitative RT-PCR. The typical gel patterns are shown (upper panels). Histograms exhibit relative levels of those RT-PCR products to that of *cyclophilin*. (B) 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)).

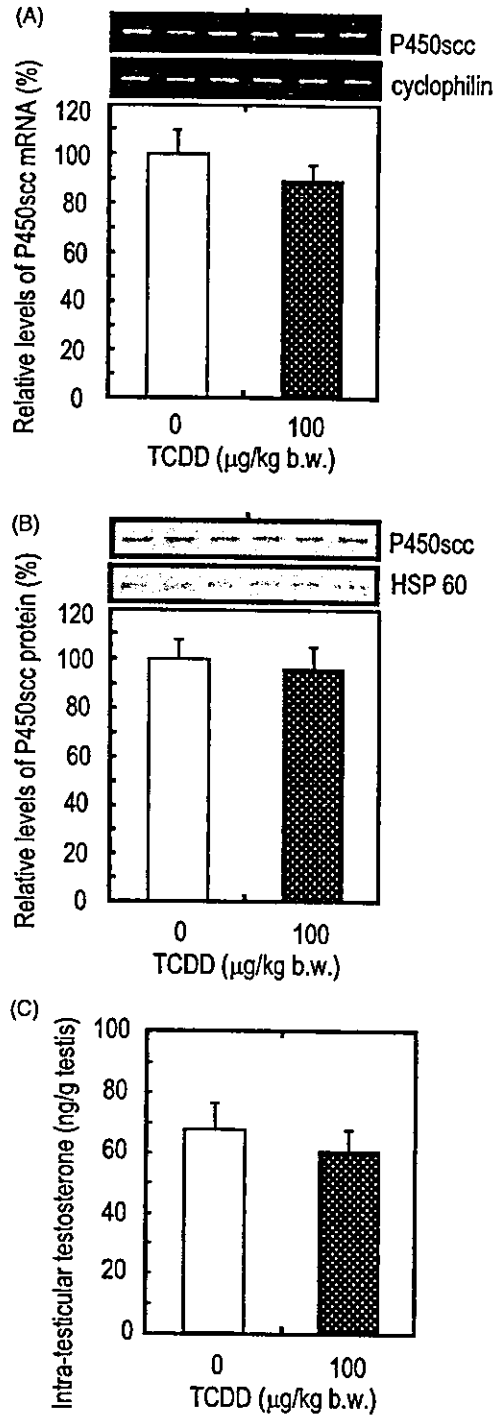

Fig. 2. Effects of TCDD on testosterone synthesis in AhR-null mouse testis. Representative signals of three mice from each groups are shown in the upper part of the figure. The values for (A) and (B) were quantified by densitometric analysis, and expressed as means ± S.E. for a total of four and six samples, respectively. (A) Effects of TCDD on P450scc mRNA levels using semiquantitative RT-PCR analysis. The histogram exhibits relative levels of P450scc to cyclophilin mRNA. (B) Effects of TCDD on P450scc production using the Western blot analysis. Results are plotted as relative levels of P450scc/HSP60 ratio. (C) Testicular testosterone concentration in AhR-null mouse exposed to TCDD. The concentration of testosterone was measured from three mice. For (A)–(C), no statistically significant difference was observed by Student's *t*-test.

3.2. *Effects of TCDD on levels of P450scc and testosterone in AhR-null mouse testis*

In mouse testis, the constitutive expression of *AhR* and *Arnt* was detected using the semiquantitative RT-PCR analysis (data not shown), suggesting that there exists an AhR-dependent signal-transduction pathway in the testis. To clarify possible involvement of AhR in the effects of TCDD on the reduction of the P450scc and concentration of ITT, we administrated TCDD to AhR-null mice at a very high dose of TCDD, 100 μg/kg bw. Semiquantitative RT-PCR analysis (Fig. 2A) and Western blot analysis using anti-P450scc antibody (Fig. 2B) showed that the amounts of mRNA and protein of P450scc were not affected in TCDD-treated AhR-null mice. The concentration

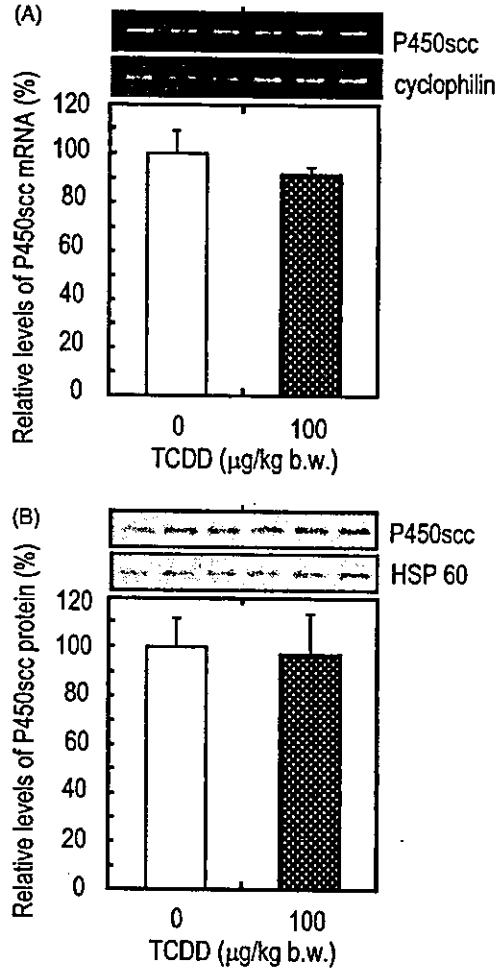

Fig. 3. Expression of *P450scc* in wild-type mouse adrenal. Representative signals of three mice from each groups are shown in the upper part of the figure. (A) Relative mRNA levels of the adrenal P450scc using semiquantitative RT-PCR analysis. The histogram exhibits relative levels of P450scc to cyclophilin mRNA. (B) Relative protein levels of the adrenal P450scc recognized by anti-P450scc antibody. Results are plotted as relative levels of P450scc/HSP60 ratio. For each Panel (A) and (B), no statistically significant difference was observed by Student's *t*-test. The values are expressed as means ± S.E. for a total of four and six samples for (A) and (B), respectively.

of ITT was not altered in TCDD-treated AhR-null mice (Fig. 2C).

3.3. Effects of TCDD on levels of P450scc in the wild-type adrenal gland

Since P450scc acts as an enzyme for side-chain cleavage of cholesterol and is situated in the mitochondrial membrane in the adrenal gland, we investigated whether the decrease in P450scc amount is a specific phenomenon in the testis (Fig. 1B) or can be observed in the adrenal gland. When we examined the amounts of adrenal P450scc mRNA and protein by the semiquantitative RT-PCR analysis and Western blot analysis using anti-P450scc antibody, respectively, no alterations in both the mRNA and protein levels of adrenal P450scc were caused by the TCDD administration (Fig. 3).

3.4. Comparison of the responses of the pituitary to TCDD with that of EB in wild-type mouse

To address the question of whether TCDD affects the hypothalamo-pituitary-gonadal (HPG) axis, we compared the possible effects of TCDD with those of EB-treated adult male mice. As a positive control, EB was given intraperi-

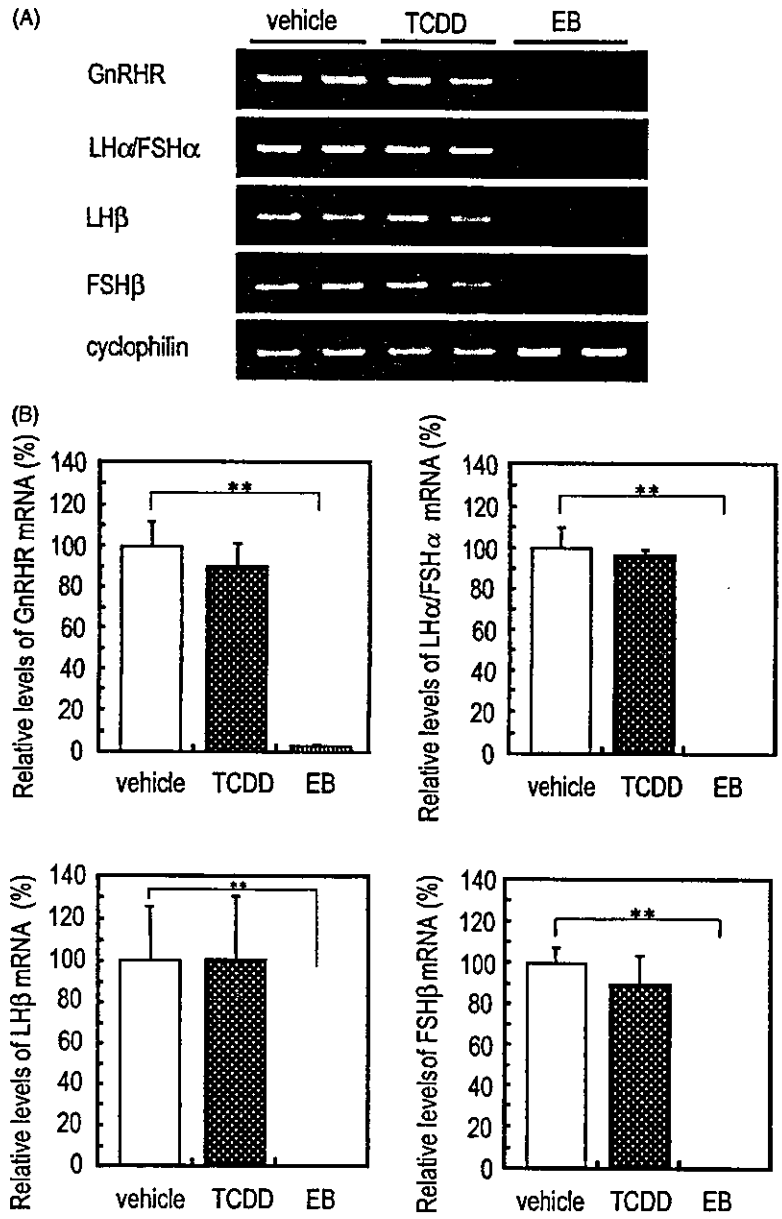

Fig. 4. Semiquantitative RT-PCR analysis of the effects of TCDD on GnRHR and gonadotropins subunits mRNA in wild-type mouse pituitary. The typical gel patterns of two individual mouse from each groups are shown (upper panels). (A) Agarose gel electrophoretic pattern for GnRHR, LHα/FSHα, LHβ, FSHβ and cyclophilin. (B) Relative intensities of PCR products to cyclophilin (means ± S.E. for three samples). A two-tailed Student's *t*-test was used to compare the means between the TCDD-treated or EB-treated and vehicle groups. Asterisks denote a significant difference from control levels ((**)$P < 0.01$). No statistically significant difference was found between the TCDD and vehiclel groups.

toneally to adult male mice at a single dose of 100 μg/kg bw, with analysis 7 days later. The semiquantitative RT-PCR was used to analyze the expression of *LHα/FSHα*, *LHβ*, *FSHβ* and *GnRHR* in the pituitary. No significant changes were found in the pituitary of TCDD-treated mice. (Fig. 4A). In contrast, in the pituitary of EB-treated mice, the levels of GnRHR mRNA was drastically decreased and that of LHα/FSHα, LHβ, or FSHβ mRNA was completely diminished (Fig. 4B).

3.5. Effects of TCDD or EB on levels of gonadotropin receptors in the testis

We examined possible effects of TCDD on the expression of two gonadotropin receptors, LHR and FSHR, in the testis. As shown in Fig. 4, mice treated with EB did not harbor detectable levels of mRNAs of the LH and FSH subunits in their testis. When TCDD was administered at a dose of 0, 0.8, 4, 20, or 100 μg/kg bw in adult mice, in TCDD-treated mouse testes, the expression of *LHR* was down-regulated in a dose-dependent manner. The mouse testis from TCDD-administered mice at a dose of 4, 20 or 100 μg TCDD/kg bw had LHR mRNA levels of approximately 63, 58, and 39% of those normally seen in control mice, respectively (Fig. 5A). In EB-treated mouse testes, the levels of LHR mRNA were significantly elevated (approximately 174%) as compared to that for control mice ($P < 0.01$) (Fig. 5B).

No significant difference in the expression of *FSHR* was observed between the TCDD-treated or EB-treated and vehicle mice by semiquantitative RT-PCR analysis (Fig. 5C). The expression of *ABP* that is induced by the FSH stimulation in Sertoli cells was not affected by TCDD treatment (Fig. 5D).

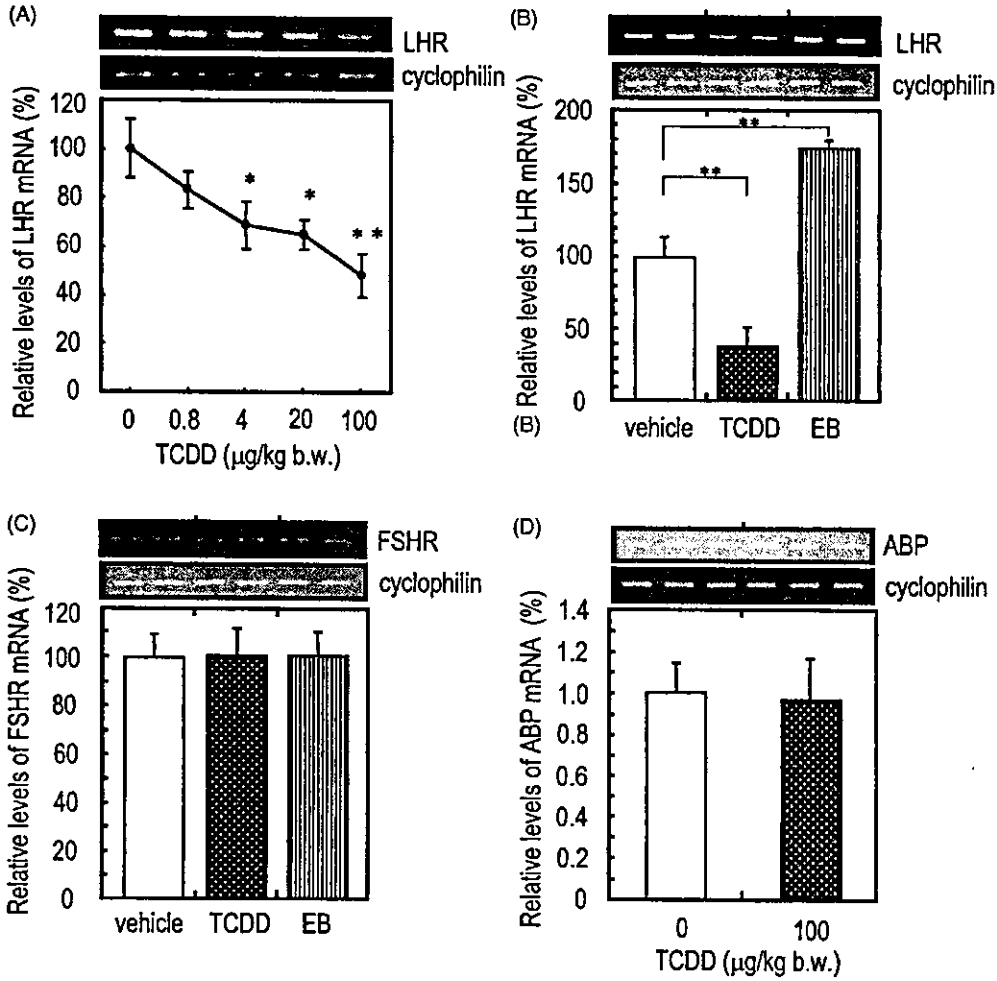

Fig. 5. Semiquantitative RT-PCR analysis of the effects of TCDD on LHR, FSHR and ABP mRNA in wild-type mouse testis. A representative gels are shown in the upper part of the histograms for (A)–(D). For these panels, values were quantified by densitometric analysis (means ± S.E. for five samples). Asterisks denote a significant difference from control levels ((*)$P < 0.05$ and (**)$P < 0.01$). Statistical difference was analyzed by ANOVA followed by Fisher's PLSD test for (A) and by two-tailed Student's *t*-test for (B)–(D). (A) Dose-related effects of TCDD on LHR mRNA (as described in Fig. 1A). (B) Relative levels of LHR mRNA. (C) Relative levels of FSHR mRNA. For (B) and (C), a two-tailed Student's t-test was used to compare the means between the TCDD- or EB-treated and vehicle groups. (D) Relative levels of ABP mRNA. Histograms for (A)–(D) exhibits relative levels of those RT-PCR products to that of cyclophilin.

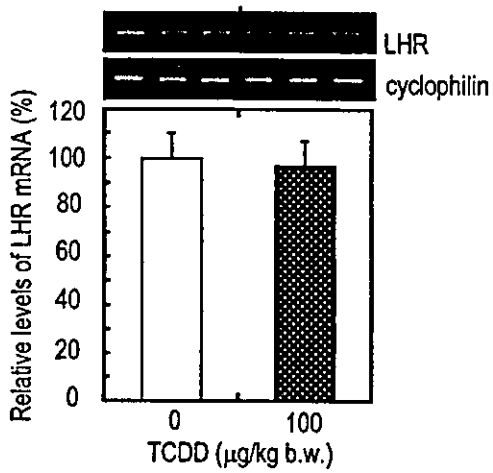

Fig. 6. Semiquantitative RT-PCR analysis of the effects of TCDD on LHR in AhR-null mouse testis. Agarose gel electrophoretic pattern of LHR and cyclophilin are illustrated in the upper part of figure. Levels of LHR mRNA relative to cyclophilin. Values are means ± S.E. ($n = 4$ per each dose). No statistical significance was found by Student's t-test.

To examine whether *LHR* expression was mediated by *AhR*, we administrated TCDD to AhR-null mice in a single dose of 100 μg/kg bw, which resulted in no alteration in the levels of LHR mRNA (Fig. 6).

4. Discussion

We performed the present study to elucidate the possible critical target of TCDD in terms of a decrease in concentrations of testosterone in vivo. It is clear that alteration in the expression of *P450scc* and *LHR* plays an important role in the TCDD-impeded production of testosterone. Impairment of steroidogenic enzyme activities caused by a high dose of TCDD has been reported in adult male rodents (Mebus et al., 1987; Moore et al., 1985; Ruangwises et al., 1991). In our experiments, the amount of testicular P450scc was reduced in a TCDD dose-dependent manner (Fig. 1), suggesting not only a suppression of catalytic activity of testicular P450scc but also a TCDD toxicity-related inhibition of the expression, whereas no changes were observed for the levels of adrenal P450scc in TCDD-treated mice (Fig. 3). These results suggest that TCDD specifically affect steroidogenesis in the testis.

The action of LH and FSH depends on both the quantity of the hormone and numbers of the specific receptors in the testis, and in the present study TCDD did not change the expression of *FSH*, *FSHR* and *ABP* (Figs. 4 and 5), which suggests that there was a normal interaction between *FSHR* and *FSH* in the TCDD-treated mouse testis. On the other hand, a variety of expression patterns were observed for the levels of *LH*, *LHR*, *P450scc*, and *StAR* in TCDD-treated mice (Figs. 1, 4 and 5). That is, the levels of LHR in TCDD-treated mice were decreased without increasing the LH synthesis in the pituitary, suggesting that Leydig cells in testis were not able to fully receive the LH pulse via LHR. In addition, no change was observed in the levels of StAR mRNA by TCDD (Fig. 1). These observations may support the notion that LHR is one of the target of TCDD.

When Leydig cells receive LH pulse stimuli, the elevation in transcription and translation of the cytochrome P450 enzymes continuously activate steroidogenesis (Payne and Youngblood, 1995). The reduction of the quantity of P450scc mRNA and protein upon TCDD exposure directly decreased the number of LHR molecules. Since the quantity of P450scc mRNA and concentration of ITT were suppressed in LHR deficient mice (Lei et al., 2001; Zhang et al., 2001), these may be similar mechanisms, in which TCDD exposure decreased the levels of *P450scc* and *LHR* in the mouse testis, as shown in the present study. The reduction of LHR mRNA was also observed in TCDD-exposed cultured ovarian granulosa cells, as assessed by nuclear run-on transcription assay (Hirakawa et al., 2000; Minegishi et al., 2003). The rates of *LHR* gene transcription directly decreased by TCDD in the female reproductive cells might shed light on the decreased gene expression of *LHR* in the testis exposed to TCDD in vivo. More functional analysis of *LHR* expression needs to be performed.

In the present study, we found a difference between the effects of TCDD and those of EB, an estradiol (E2) agonist, on the pituitary–gonadal axis. It is well established that testosterone synthesis is regulated by negative feedback regulation: in males, when the serum concentration of testosterone increases, the E2, testosterone metabolite, is also increased accordingly. E2 affects the hypothalamo–pituitary axis and subsequently causes a reduction of LH. This lack of a LH pulse then leads to the suppression of testosterone production. When EB was administered to mice, it resulted in a severe impairment of the expression of *GnRHR* and LH with a significant increase in the expression of *LHR* (Figs. 4 and 5), which suggests that EB impedes production of ITT. In fact, EB exposure has been reported to result in the reduction of the concentration of LH and testosterone in serum, thus leading to a decrease in ITT (Hossaini et al., 2003). On the other hand, exposure to TCDD caused reduction of LHR and concentrations of ITT (Figs. 1 and 5) without increasing the expression of *LH* (Fig. 4), the results of which are consistent with the observation in that TCDD did not affect the LH secretion without affecting GnRH sensing functions in the pituitary (Petroff et al., 2000). These observations demonstrate that TCDD has a different target than that of EB in the HPG axis.

In the LHR-null mice, the serum LH contents were found to increase when the ITT contents were reduced (Lei et al., 2001; Zhang et al., 2001). In TCDD-treated wild-type mice, however, no changes were observed in the synthesis of LH when the levels of LHR mRNA was reduced (Figs. 4 and 5). Moreover, in TCDD treated-castrated wild-type rats, TCDD was not able to inhibit the LH synthesis and its secretion process (Bookstaff et al., 1990b), which is consistent with the present results that TCDD did not affect the levels of LHα/FSHα, and LHβ mRNA (Fig. 5).

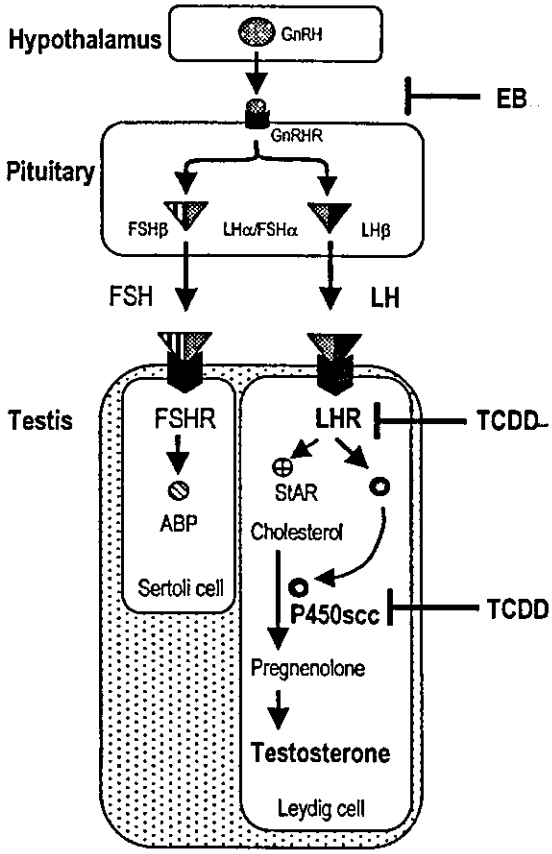

Fig. 7. The proposed mechanism how TCDD and EB affect testosterone production.

Finally, we demonstrated (Fig. 7) that TCDD suppressed testicular *P450scc* and *LHR* expression in an AhR-dependent manner. The down-regulation of these genes is the trigger that leads to the suppression of testosterone production in vivo. Based on the present and earlier study results, we could speculate that TCDD treatment: (1) suppresses the expression of *LHR* and *P450scc* in the testis with the subsequent suppression of ITT production; (2) does not increase the synthesis of LH from pituitary; and (3) prevents an increase in serum LH concentrations. The present findings shed light to the clarification of perturbation and toxicity mechanism of TCDD in the androgen homeostasis in the rodent males.

Acknowledgements

The authors thank Miss Satoko Takahashi (University of Tsukuba), and Miss Fumi Kido (University of Tsukuba) for their excellent technical help in this experiment. This work was supported in part by grants from CREST, JST (C.T.), the Ministry of Health, Labor and Welfare (C.T.), the Ministry of Education, Science, Sports and Culture (C.T.).

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