across eight sections for each fetus, with one fetus per litter and six litters per treatment group.

Radioimmunoassay. Total T_4 was measured in 5 µL of rat serum using a barbital buffer system. Briefly, each assay tube contained 100 µL barbital buffer (0.11 M barbital, pH 8.6; 0.1% wt/vol 8-anilino-1-naphthalenesulfonic acid ammonium salt; 15% bovine γ-globulin Cohn fraction II; 0.1% gelatin), 100 µL anti-T_4 (rabbit, diluted to provide a final concentration of 1:30,000; Sigma, St. Louis, MO), and 100 µL ^{125}I-labeled T_4 (Perkin-Elmer/NEN; Boston, MA). Standards were prepared from T_4 (Sigma) measured using a Cahn electrobalance; standards were run in triplicate, whereas samples were run in duplicate. Standards were calibrated to measure serum T_4 levels from 0.4 µg/dL to 25.6 µg/dL. Tubes were incubated at 37°C for 30 min and then chilled on ice for 30 min. Bound counts were precipitated by adding 300 µL ice-cold polyethylene glycol 8000 (20% wt/wt; Sigma). Tubes were centrifuged at 1800 × *g* for 20 min at 4°C; the supernatant was then aspirated and the pellet counted in a gamma counter (Packard Cobra II; Global Medical Instrumentation, Inc., Albertville, MN). The assay was run at 40–50% binding; nonspecific binding was generally < 8%. The assay was validated for rat serum by demonstrating parallelism between the standard curve and a dilution series of rat serum. The two slopes did not vary significantly as evaluated by *t*-test for two slopes (data not shown). The variability within the assay was determined by running 10 replicates of three different standards that represent a low, medium, and high value on the standard curve. The coefficients of variance were 0.9% for 0 ng/mL; 4.7% for 3.2 µg/dL, and 3.8% for 25.6 µg/dL. All experimental samples were evaluated in a single assay.

Total triiodothyronine (T_3) was measured according to the manufacturer's instructions using a T_3 RIA kit (ICN Diagnostics, Costa Mesa, CA). This assay was performed at 49% binding with detection limits of 50–800 ng/dL. All samples were evaluated in a single assay.

Isolation of hepatic nuclei. Adult male Sprague-Dawley rats were euthanized with carbon dioxide and perfused with ice-cold saline through the aorta until the liver was cleared of blood. Twenty grams of liver was then washed in 3 mM $MgCl_2$ and 0.14 M NaCl, minced, and homogenized in 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.32 M sucrose using a motor-driven Teflon mortar and glass pestle. The homogenate was centrifuged at 600 × *g* for 10 min, and the crude nuclear pellet was resuspended in 3 mM $MgCl_2$, 1 mM DTT, and 1.8 M sucrose. After centrifugation at 53,000 × *g* for 45 min, the nuclei were resuspended in binding buffer (3 mM $MgCl_2$; 1 mM DTT; 20 mM Tris HCl, pH 7.6; 0.32 M sucrose; and 0.3% bovine serum albumin) and stored at –80°C.

TH binding assay. For saturation analysis, nuclear isolates frozen in binding buffer were thawed on ice, and triplicate aliquots (~0.1 g of the original liver) were incubated with increasing concentrations of ^{125}I-T_3 (1×10^{-10} to 8×10^{-9} M; 3,300 µCi/µg; NEN, Boston, MA) for 30 min at 37°C. Nonspecific binding was determined at each concentration of ^{125}I-T_3 (and each competitor concentration) by performing the assay as described in a parallel set of tubes that included the addition of 10,000-fold excess cold T_3 (final concentration 1×10^{-6} M). The reaction was terminated by placing samples on ice and by adding binding buffer/1% Triton X-100. Samples were centrifuged at 13,000 × *g* for 10 min, the supernatant was discarded, and the nuclear pellet was washed in 1 mL binding buffer. The bottom of the microfuge tube

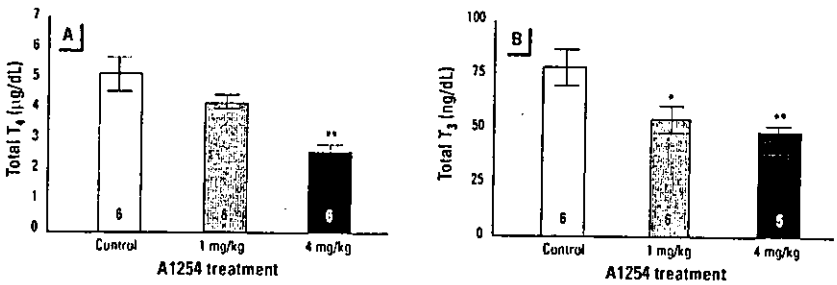

Figure 2. Effect of A1254 treatment on serum concentrations of total T_4 (*A*) and total T_3 (*B*) in dams at the time they were sacrificed on GD16. Bars represent mean ± SEM; number of animals in each group is shown within each bar. See Materials and Methods for treatment details.
*$p < 0.05$; **$p < 0.01$ (significantly different from control group using Bonferroni's *t*-test after one-way ANOVA).

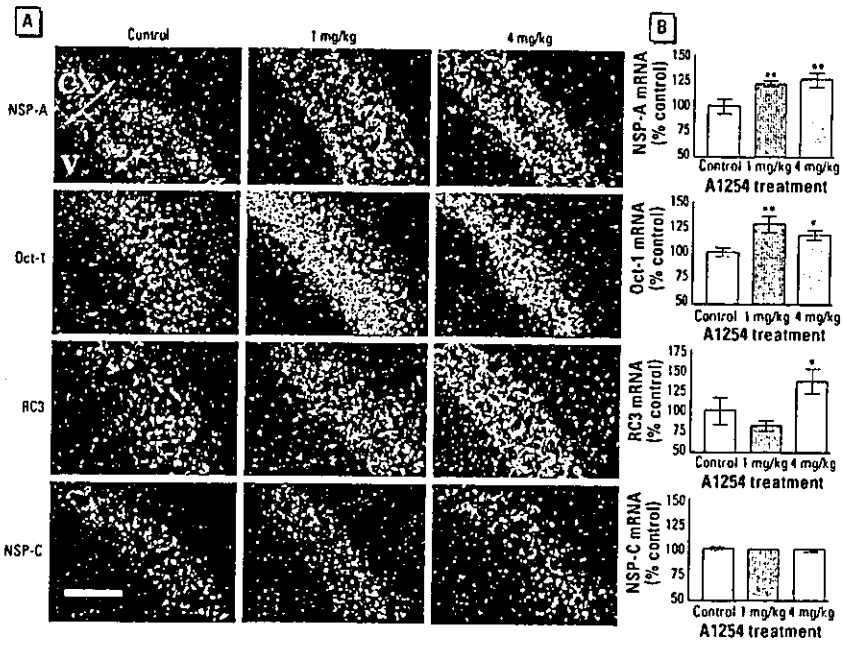

Figure 3. Effect of A1254 treatment on the levels and patterns of NSP-A, Oct-1, RC3/neurogranin, and NSP-C mRNA expression in the GD16 fetal cortex. (*A*) Representative pseudocolor autoradiograms obtained after *in situ* hybridization (pseudocolor signal intensity: red > yellow > blue > black). (*B*) Bar graphs showing the relative abundance of mRNA expression as reflected by the mean ± SEM film density (Oct-1 and NSP-C), area (NSP-A), or integrated density (RC3) and are presented as percent control for the purpose of illustration. Abbreviations: CX, cortex; V, lateral ventricle; VZ, ventricular zone. Measurements were taken from the VZ for NSP-A and revealed that the relative area of mRNA expression was significantly elevated in the 1 mg/kg and 4 mg/kg treatment groups. The pattern of Oct-1 expression was not specific to the VZ, and measurements taken in the CX showed a relative increase in the density of Oct-1 mRNA in both the 1 mg/kg and 4 mg/kg treatment groups. Measurements taken for RC3/neurogranin were also taken in the CX, and when density measurements were normalized with respect to area (integrated density), an increase in the level of mRNA expression was detected in the 4 mg/kg A1254 treatment group. All CX measurements revealed that NSP-C mRNA levels did not differ among treatment groups. Bar = 50 µm.
*$p < 0.05$. **$p < 0.01$ (significantly different from control using Bonferroni's *t*-test after one-way ANOVA).

containing the pellet was cut off and placed in a 14 × 70 mm test tube, which was counted in a gamma counter (Packard Cobra II). For competitive binding assays, isolated nuclei were incubated with a final concentration of 1×10^{-10} M ^{125}I-T_3 and increasing concentrations of competitors (TR agonists: T_3, T_4, Triac (triiodothyroacetic acid), and Tetrac (tetraiodothyroacetic acid, 1×10^{-12} M to 1×10^{-9} M; PCBs, 1×10^{-9} M to 1×10^{-3} M). For noncompetitive binding assays, isolated nuclei were incubated with 1×10^{-10} M ^{125}I-T_3 and increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 alone or increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 in the presence of 2×10^{-6} M of competitors. Nonspecific binding was established as described above.

Statistical analysis. Results were analyzed using a one-factor analysis of variance (ANOVA), and post hoc tests, where appropriate, were performed by Bonferroni's *t*-test, where the mean squared error term in the ANOVA table was used as the point estimate of the pooled variance (SuperAnova Software; Abacus Concepts, Inc., Berkeley, CA). A test for outliers was performed on all data; none were identified. Some samples were lost in processes; therefore, there are some unequal cell sizes.

Results

Dams. Exposure to A1254 significantly reduced circulating levels of total T_4 and total T_3 in dams (T_4: $F_{(2,15)} = 11.031$, $p = 0.0011$; T_3: $F_{(2,14)} = 5.772$, $p = 0.0142$; Figure 2). Post hoc analysis using Bonferroni's *t*-test revealed that dams treated daily with 4 mg/kg A1254 exhibited T_4 levels that were significantly lower than those of control animals (Figure 2A). There was a trend in animals treated with 1 mg/kg A1254 to exhibit lower circulating levels of T_4, but this did not reach statistical significance (Figure 2A). Moreover, animals treated with either 1 or 4 mg/kg A1254 exhibited significantly lower levels of circulating T_3 compared with control animals (Figure 2B).

Fetal brains. Quantitative analysis of film autoradiograms after *in situ* hybridization revealed that PCB exposure selectively affected TH-responsive genes in the fetal cortex (Figure 3). We focused on four different genes. RC3/neurogranin expression was significantly higher in the cortex of fetuses derived from dams treated with 4 mg/kg A1254 ($F_{(2,15)} = 5.423$, $p = 0.0169$). NSP-A expression was significantly elevated in fetuses derived from dams treated with either 1 or 4 mg/kg A1254 ($F_{(2,13)} = 8.212$, $p = 0.0049$),

Table 2. TH receptor agonists, parent PCB congeners, $MeSO_2$-PCB metabolites, and hydroxylated PCB metabolites tested in competitive TR binding assays.

Competitor	Abbreviation	IUPAC nomenclature	K_i	± CV
Triiodothyronine	T_3	L-3,3′,5-Triiodothyronine	8.37×10^{-10} M	12.70%
Thyroxine	T_4	L-3,3′,5,5′-Tetraiodothyronine	3.74×10^{-9} M	14.40%
Triiodothyroacetic acid	Triac	3,3′,3-Triiodothyroacetic acid	1.29×10^{-8} M	17.30%
Tetraiodothyroacetic acid	Tetrac	3,3′,5,5′-Tetraiodothyroacetic acid	2.37×10^{-7} M	18.40%
Parent PCBs	PCB-77	3,3′,4,4′-TetraCB	ND	NA
	PCB-105	2,3,3′,4,4′-PentaCB	ND	NA
	PCB-118	2,3′,4,4′,5-PentaCB	ND	NA
	PCB-126	3,3′,4,4′,5-PentaCB	ND	NA
	PCB-138	2,2′,3,4′,4′,5′-HexaCB	ND	NA
	PCB-153	2,2′,4,4′,5,5′-HexaCB	ND	NA
$MeSo_2$-PCBs	3-$MeSO_2$-PCB-49	3-$MeSO_2$-2,2′,4′,5-tetraCB	ND	NA
	4-$MeSO_2$-PCB-49	4-$MeSO_2$-2,2′,4′,5-tetraCB	ND	NA
	3-$MeSO_2$-PCB-70	3-$MeSO_2$-2,3′,4′,5-tetraCB	ND	NA
	4-$MeSO_2$-PCB-70	4-$MeSO_2$-2,3′,4′,5-tetraCB	ND	NA
	3-$MeSO_2$-PCB-87	3-$MeSO_2$-2,2′,3′,4′,5-pentaCB	ND	NA
	3-$MeSO_2$-PCB-101	3-$MeSO_2$-2,2′,4,5,5′-pentaCB	ND	NA
	4-$MeSO_2$-PCB-101	4-$MeSO_2$-2,2′,4,5,5′-pentaCB	ND	NA
	3-$MeSO_2$-PCB-132	3-$MeSO_2$-2,2′,3,4′,5,5-hexaCB	ND	NA
	3-$MeSO_2$-PCB-141	3-$MeSO_2$-2,2′,3′,4′,5,5′-hexaCB	ND	NA
	3-$MeSO_2$-PCB-149	3-$MeSO_2$-2,2′,4′,5,5,6-hexaCB	ND	NA
	4-$MeSO_2$-PCB-149	4-$MeSO_2$-2,2′,4′,5,5,6-hexaCB	ND	NA
	4-$MeSO_2$-PCB-52	4-$MeSO_2$-2,2′,4,4′-tetraCB	ND	NA
	3-$MeSO_2$-PCB-77	3-$MeSO_2$-3′,4,4′,5-tetraCB	ND	NA
	3-$MeSO_2$-PCB-105	3-$MeSO_2$-2′,3′,4,4′,5-pentaCB	ND	NA
	3-$MeSO_2$-PCB-118	3-$MeSO_2$-2′,4,4′,5,5′-pentaCB	ND	NA
	3-$MeSO_2$-PCB-156	3-$MeSO_2$-2′,3′4,4′,5,5′-hexaCB	ND	NA
Hydroxylated PCBs	4′-OH-PCB-3	4′-OH-4-monoCB	ND	NA
	4′-OH-PCB-9	4′-OH-2,5-diCB	ND	NA
	4-OH-PCB-14	4′-OH-3′,5′-diCB	ND	NA
	4′-OH-PCB-12	4′-OH-3,4-diCB	ND	NA
	4-OH-PCB-30	4′-OH-2,4,6-triCB	ND	NA
	4′-OH-PCB-34	4′-OH-2,3′,5′-triCB	ND	NA
	4′-OH-PCB-36	4′-OH-3,3′,5-triCB	ND	NA
	4′-OH-PCB-20	4′-OH-2,3,3′-triCB	ND	NA
	4′-OH-PCB-35	4′-OH-3,3′,4-triCB	ND	NA
	4-OH-PCB-39	4-OH-3,4′,5-triCB	ND	NA
	4′-OH-PCB-58	4′-OH-2,3,3′,5′-tetraCB	ND	NA
	4′-OH-PCB-72	4′-OH-2,3′,5,5′-tetraCB	ND	NA
	4′-OH-PCB-106	4′-OH-2,3,3′,4,5-pentaCB	ND	NA
	4′-OH-PCB-112	4′-OH-2,3,3′,5,6-pentaCB	ND	NA
	4′-OH-PCB-159	4′-OH-2,3,3′,4,5,5′-hexaCB	ND	NA
	4′-OH-PCB-165	4′-OH-2,3,3′,5,5′,6-hexaCB	ND	NA
	3′,4′-(di)OH-PCB-12	3′,4′-OH-3,4-diDB	ND	NA
	3′,4′-(di)OH-PCB-3	3′,4′-OH-4-monoCB	ND	NA
	3′-OH-PCB-3	3′-OH-4-monoCB	ND	NA
	2′-OH-PCB-3	2′-OH-4-monoCB	ND	NA

Abbreviations: CB, chlorinated biphenyl; IUPAC, International Union of Pure and Applied Chemistry; NA, not applicable; ND, no detectable binding.

as was Oct-1 ($F_{(2,14)} = 5.399$, $p = 0.0183$). In contrast, NSP-C expression in the GD16 cortex was not affected by PCB exposure ($F_{(2,15)} = 0.202$, $p = 0.819$; not significant).

TH receptor binding. To test the hypothesis that individual PCB congeners may bind to TRs to produce the observed effects on gene expression *in vivo*, we tested a number of parent PCBs and metabolites for their ability to bind to the TR (Table 2). To validate the assay, we first performed a saturation analysis and established that T_3 bound to TRs in isolated hepatic nuclei with a K_d of $9.7 \times 10^{-10} \pm 2.02 \times 10^{-10}$ M (Figure 4). We then tested the ability of T_3 and other TR agonists (T_4, Triac, and Tetrac) to displace ^{125}I-T_3 from TRs in hepatic nuclei (Figure 5A); using the K_d obtained from saturation analyses, a specific K_i was calculated for each compound (Table 2). However, none of the tested parent PCB congeners, hydroxylated metabolites, or $MeSO_2$ metabolites significantly displaced ^{125}I-T_3 in this assay (Figure 5B). Similarly, noncompetitive binding analysis revealed that parent PCB congeners, grouped according to their *ortho*-substitution pattern, did not alter the affinity of T_3 for TRs in isolated nuclei (Figure 6).

Discussion

The present study demonstrates that the commercial PCB mixture A1254 significantly reduces serum TH levels, T_4, and T_3, in pregnant rats on GD16. This developmental time occurs before the onset of thyroid function in the fetus (Fisher et al. 1977); therefore, it is reasonable to propose that this PCB-induced decrement in maternal TH would cause a reduction in the expression of genes positively regulated by TH. However, we found that PCB exposure up-regulated the expression of genes that are positively regulated by TH. These findings indicate that PCBs can activate TRs, perhaps directly, and the implication is important because inappropriate activation of TRs in the developing brain may produce adverse consequences on brain development (Kopelman 1983; Rastogi and Singhal 1976, 1979; Zoeller 2003).

Our finding that A1254 decreased circulating levels of TH in pregnant rats is consistent with previous studies showing that exposure to A1254, or specific PCB congeners, causes a reduction in circulating levels of total T_4 in pregnant rats (Meerts et al. 2002; Morse et al. 1993, 1996). Additionally, we found that serum total T_3 was also reduced in the dams by A1254, indicating the degree to which serum T_4 was reduced, because nearly 80% of circulating T_3 is derived from peripheral deiodination of T_4 (Taurog 2000). Although PCB exposure reduced serum T_4 in the dams, several descriptive measures of hypothyroidism were not altered. For example, maternal body weight, weight gain, litter size, and pup weight were all unaffected by PCB treatment in this experiment (data not shown), similar to results of our previous study (Zoeller et al. 2000). Therefore, like others, we observed a discrepancy between the ability of PCB exposure to lower serum TH levels and its ability to produce symptoms of hypothyroidism.

The present finding that fetuses derived from A1254-treated dams exhibited a significant increase in RC3/neurogranin and Oct-1 expression represents strong evidence that PCBs can produce TH-like effects in the fetal brain because maternal TH increases the expression of these genes in the fetal brain (Dowling et al. 2000; Dowling and Zoeller 2000). Considering that A1254 exposure produced a significant reduction in circulating T_4 and T_3 in the dam, this finding is fully consistent with the hypothesis that PCBs can directly activate TRs in the fetal brain. Moreover, A1254 exerted selective effects on the expression of TH-responsive genes because NSP-C, which is not influenced by TH in the fetus (Dowling et al. 2000, 2001), was not affected by A1254. Furthermore, previous work in our laboratory has demonstrated that A1254 increases the expression of myelin basic protein, a known TH-responsive gene (Farsetti et al. 1991; Marta et al. 1998; Rodriguez-Pena et al. 1993), in the cerebellum and RC3/neurogranin in the forebrain of postnatal rats, despite the finding that these pups exhibited a

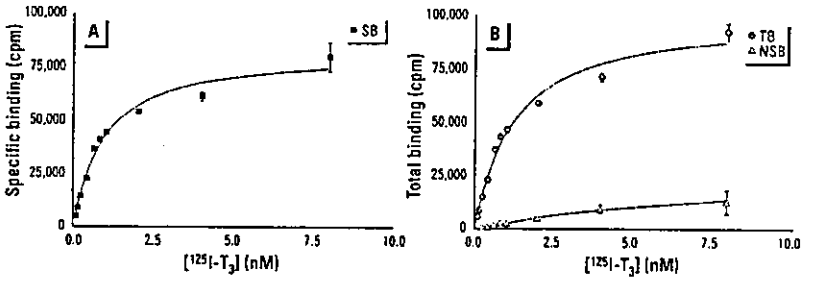

Figure 4. (*A*) Saturation analysis of ^{125}I-T_3 specific binding (SB) to TRs in nuclei isolated from rat liver tissue, and (*B*) total binding (TB) obtained by incubating hepatic nuclei with increasing concentrations (1×10^{-10} to 8×10^{-9}) ^{125}I-T_3 ($n = 3$; see "Materials and Methods" for assay conditions). NSB, nonspecific binding.

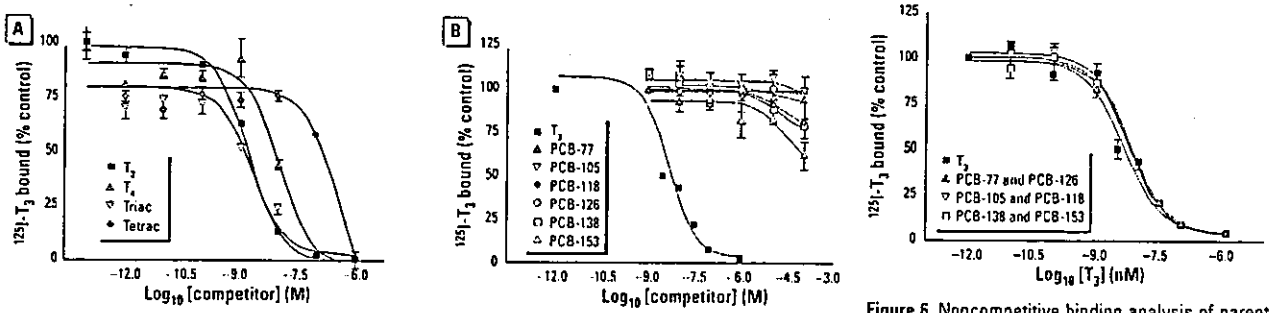

Figure 5. Competitive binding of known TH receptor agonists (*A*) and parent PCB congeners (*B*) to isolated hepatic nuclei. In (*A*), rat liver nuclear extracts were incubated with 1.0 nM ^{125}I-T_3 and increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3, T_4, Triac, or Tetrac; TR agonists displayed an expected order of binding affinity to isolated nuclei (T_3 = Triac > T_4 >> Tetrac). In (*B*), isolated nuclei were incubated with 1.0 nM ^{125}I-T_3 and increasing concentrations (1×10^{-9} M to 1×10^{-3} M) of one of the parent PCB congeners; no parent PCB congener exhibited significant binding to isolated nuclei. (*B*) is representative of results obtained from assays that tested hydroxylated and $MeSO_2$-PCB metabolites for their ability to displace ^{125}I-T_3 from TRs in hepatic nuclei. These compounds also showed no significant binding to nuclear extracts. All curves are were obtained from results of a single experiment and are representative of three separate experiments.

Figure 6. Noncompetitive binding analysis of parent PCB congeners binding to isolated hepatic nuclei. Assays were performed to determine whether PCBs present in A1254 were able to alter the affinity TRs for T_3. Isolated nuclei were incubated with 1.0 nM ^{125}I-T_3, and increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 alone or with increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 in the presence of 20 μM of the PCB competitor. These PCBs were unable to alter the established K_i of T_3 for TRs in hepatic nuclei.

severe reduction in the circulating levels of TH (Zoeller et al. 2000). Taken together, these data indicate that A1254 can exert agonistic effects on a variety of positively regulated TH-responsive genes in different brain regions at different developmental times.

Although A1254 produced TH-like effects on the expression of RC3/neurogranin and on Oct-1 in the fetal cortex in the present study, it did not exert these effects on NSP-A expression. We previously found that NSP-A expression is significantly increased in the brain of fetal rats derived from hypothyroid dams (Dowling et al. 2000, 2001). Therefore, the present finding that NSP-A expression is increased in the cortex of fetuses exposed transplacentally to A1254 suggests that the expression of this gene is responding to low maternal T_4 in PCB-treated dams, not to agonistic actions of PCBs on TRs. Previously, we showed that T_4 treatment of hypothyroid dams did not restore NSP-A expression in the fetal cortex (Dowling et al. 2001). Because T_4 was provided for only a short time, the interpretation was that the duration of T_4 treatment was not sufficient to produce a significant reduction in cellular levels of NSP-A mRNA. However, Chan et al. (2003) have recently reported that NSP-A expression is not directly sensitive to TH in N-Tera-2 cells, indicating that NSP-A may not be directly regulated by TH. If maternal hypothyroidism increases NSP-A expression indirectly, and NSP-A is not directly regulated by TH, then our present results indicate that PCBs produce effects on the fetal brain by exerting direct TH-like effects as well as by inducing low maternal TH.

Considering these findings, we tested a number of PCB congeners and specific metabolites for their ability to bind to TRs using a well-established binding assay (DeGroot and Torresani 1975). We found that neither the parent PCB congeners nor the hydroxylated or $MeSO_2$ metabolites significantly displace T_3 from rat hepatic nuclei. It is not likely that these observations are false negatives because the observed K_i for several control ligands, including T_3, T_4, Tetrac, and Triac, were all within the published range (Evans and Braverman 1986; Goslings et al. 1976; Ichikawa and DeGroot 1987). In addition, we demonstrated in preliminary studies that the PCB diluent (dimethyl sulfoxide) does not interfere with the assay; moreover, we used different diluents in initial studies and obtained results that did not differ from those reported here. Thus, our finding that individual PCB congeners or their metabolites do not displace T_3 from its receptor indicates that these compounds do not interact with the TR in a competitive manner.

We employed hepatic nuclei to test whether individual PCB congeners could bind to rat TRs because the TR isoforms expressed in hepatocytes are also the predominant isoforms expressed in the fetal cortex (Bradley et al. 1992; Nakai et al. 1988). Therefore, the observation that individual PCB congeners did not displace T_3 from liver nuclei suggests that they also do not displace T_3 from TRs in the fetal cortex. However, Cheek et al. (1999) demonstrated that several hydroxylated PCB metabolites bind to the human TRβ1, although the affinity for the TR was reported to be 10,000-fold lower than that of T_3. We evaluated two of these metabolites, 4′-OH-PCB-14 and 4′-OH-PCB-106, but did not find significant binding to the TRs in rat hepatic nuclei. These two studies may differ in their findings because we used TR isoforms from a different species, or because both TRα1 and TRβ1 are expressed in hepatocytes (Nakai et al. 1988). Specifically, it is possible that we may not have observed significant T_3 displacement if a PCB congener binds to only one of the two TRs with low affinity.

There are two major implications of the present study. First, the observation that PCB exposure selectively alters gene expression in the fetal cortex strongly suggests that PCBs can exert deleterious effects on fetal brain development regardless of the mechanism by which this effect is mediated. Because we used TH-responsive genes as end points for this study, it is likely that the observed effects reflect the ability of PCBs to disrupt TH action in the fetal brain. It will be important to determine whether PCB exposure can interfere with neurodevelopmental events by interfering with TH action.

The second major implication of our present results is that PCBs do not appear to bind to TRs in a competitive manner. We were surprised to find no individual PCB congeners or metabolites that exhibited strong binding to TRs, especially considering previous speculation about this (McKinney et al. 1987; McKinney and Waller 1998; Porterfield 1994, 2000; Porterfield and Hendry 1998). However, there is evidence that PCB congeners can affect TR activation without displacing T_3. Specifically Iwasaki et al. (2002) showed that 4′-OH-PCB-106 suppressed T_3-induced transactivation by TR in various cell lines. This appeared to be specific to the TR because it did not suppress glucocorticoid receptor–mediated transactivation. In addition, they showed that this PCB congener suppressed the ability of the TR to recruit the coactivator SRC-1. Because we found that this hydroxylated PCB did not displace T_3 from rat TRs in the present study, these observations strongly suggest that PCBs can directly alter TR action by a mechanism that is not well understood.

Our failure to identify individual PCBs that can bind with high affinity to the rat TRs requires alternate explanations for their effects on TH-responsive genes and developmental events. Individual PCB congeners may alter TH metabolism by tissue deiodinases, thereby changing the amount of hormone available to the TR. Previous studies indicate that PCBs can increase type-2 deiodinase activity in the adult (Hood and Klaassen 2000) and fetal (Meerts et al. 2002; Morse et al. 1996) rat brain. Moreover, PCBs are also known to affect second messenger signaling in the brain by affecting calcium homeostasis, receptor-mediated inositol phosphate production, and translocation of protein kinase C (Kodavanti et al. 1993, 1994). In addition, PCBs can produce toxic effects by binding either to the aryl hydrocarbon receptor (Safe 1990) or the ryanodine receptor (Schantz et al. 1997; Wong et al. 1997). These studies demonstrate that PCBs, especially as a mixture such as A1254, clearly produce multiple effects. However, there is no evidence that these other mechanisms of PCB action can exert specific effects on TH-responsive genes.

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PCB類による甲状腺ホルモンかく乱作用とその作用機構：動物種差

加藤　善久*[1]，木村　良平[1]，山田　静雄[1]，出川　雅邦[2]

[1]静岡県立大学薬学部薬剤学教室，[2]衛生化学教室　〒422-8526 静岡市谷田52-1

Species differences among mice, hamsters, rats and guinea pigs in PCB-induced alteration of serum thyroid hormone level

Yoshihisa Kato[1], Ryohei Kimura[1], Shizuo Yamada[1] and Masakuni Degawa[2]

[1]Department of Biopharmacy, [2]Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1, Yada, Shizuoka 422-8526, Japan

Summary

In the present study, we investigated the mechanism for the decrease in levels of serum thyroid hormones, especially thyroxine (T_4), by polychlorinated biphenyls (PCBs) such as Kanechlor-500 (KC500), 2,2',4',5,5'-pentachlorobiphenyl (PentaCB), and 2,2',3',4',5,6-hexachlorobiphenyl (HexaCB), and studied species differences among mice, hamsters, rats, and guinea pigs in the PCB effect. Significant decrease in serum total T_4 level by KC500 was observed in all four species. On the other hand, there were differences in the level of decrease of serum total T_4 level by PentaCB and HexaCB. Differences in the level of hepatic methylsulfonyl-PCB metabolites of KC500, PentaCB and HexaCB, which were thought to be associated with the PCB-toxic effects, did not necessarily correlate with the magnitude of decrease in serum total T_4 level. Likewise, the induction of UDP-glucuronosyltransferases (T_4-UDP-GT) toward T_4 by PCB did not necessarily correlate with the decrease in serum T_4 level in the animals used. Further studies on transthyretin (TTR) and serum T_4-transporter suggested that decrease in serum total T_4 level induced by PCB occurred not only by induction of T_4-UDP-GT but also by the alteration of levels of T_4-TTR binding and hepatic T_4-transporter. In addition, species difference in the decrease of serum total T_4 was associated with various PCB-induced total effects, including induction of T_4-UDP-GT, decrease in T_4-TTR binding level, the increase of hepatic thyroid hormone transporter, and other thyroid function correlates.

Keywords: species differences, polychlorinated biphenyls, thyroid hormones, UDP-glucuronosyltransferases, transthyretin

緒　　言

Polychlorinated biphenyl (PCB) は，多くの野生生物の組織のみならず，ヒトの血液，母乳，肝臓，脂肪組織などにも見出され，生体に対する影響が懸念されている (ATSDR, 2000; Hansen, 1999)．ラットでは，すでに，PCB投与により血中T_4濃度が低下することが報告され (Table 1)，ヒトにおいてもPCB曝露により甲状腺ホルモン（サイロキシン (T_4)，3,5,3'-トリヨードサイロニン (T_3)）の撹乱が引き起こされている可能性が指摘されている (Koopman-Esseboom et al., 1994; Jacobson et al., 1990)．

一般に，PCBによるラットでの血中T_4濃度の低下は，肝臓のUDP-グルクロン酸転移酵素 (UDP-GT) が誘導

* E-mail: kato@ys7.u-shizuoka-ken.ac.jp

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Table 1 Effects of PCB and TCDD on serum thyroid hormone levels

Treatment	Animals	T_4	T_3	TSH	Refarences
2,4,4'-trichlorobiphenyl	Sprague-Dawley rats	→	→	n.t.	Ness et al., 1993
2,3',4,4',5-pentachlorobiphenyl	"	↓	→	n.t.	"
2,2',4,4',5,5'-hexachlorobiphenyl	"	↓	→	n.t.	"
3,3',4,4'-tetrachlorobiphenyl	"	↓	→	→	Seo et al., 1995
3,3',4,4',5-pentachlorobiphenyl	"	→	→	→	"
3,3',4,4',5-pentachlorobiphenyl	"	↓	n.t.	n.t.	van Birgelen et al., 1995
2,3,3',4,4',5-hexachlorobiphenyl	"	↓	n.t.	n.t.	"
TCDD	"	↓	n.t.	n.t.	"
Aroclor 1254	"	↓	↓	↑	Barter and Klaassen, 1994
"	"	↓	↓	↑	Liu et al., 1995
3,3',4,4',5-pentachlorobiphenyl	"	↓	↓	→	Desaulniers et al., 1999
2,2',4,4',5,5'-hexachlorobiphenyl	"	↑	n.t.	→	"
3,3',4,4',5-pentachlorobiphenyl	Long-Evans rats	↓	n.t.	n.t.	Craft et al., 2002
"	C57BL/6J mice	→	n.t.	n.t.	"
2,2',4,4',5,5'-hexachlorobiphenyl	Long-Evans rats	↓	n.t.	n.t.	"
"	C57BL/6J mice	↓	n.t.	n.t.	"

PCB: polychlorinated biphenyl, TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, n.t.: not tested.

Table 2 Effects of KC500, PentaCB and HexaCB on serum total T_4, free T_4, total T_3 and TSH levels in mice, hamsters, rats and guinea pigs

Treatment	Animals	Total T_4	Free T_4	Total T_3	TSH
KC500	Mice	43% ↓	44% ↓	→	→
	Hamsters	71% ↓	57% ↓	→	→
	Rats	77% ↓	76% ↓	→	→
	Guinea pigs	74% ↓	76% ↓	60% ↓	→
PentaCB	Mice	42% ↓	n.t.	42% ↓	→
	Hamsters	34% ↓	n.t.	→	→
	Rats	46% ↓	n.t.	→	→
	Guinea pigs	→	n.t.	→	→
HexaCB	Mice	39% ↓	n.t.	→	→
	Hamsters	→	n.t.	→	→
	Rats	→	n.t.	→	→
	Guinea pigs	→	n.t.	→	→

Animals were killed at 4 days after the administration of KC500 (37.5 mg/kg, ip), PentaCB (11 mg/kg, ip) or HexaCB (19 mg/kg, ip). Results are expressed as the mean for 5-6 animals. n.t.: not tested.

され，T_4のグルクロン酸抱合化が促進．そして，胆汁排泄が高まることによると考えられている（Barter and Klaassen, 1994; Schuur et al., 1997; van Birgelen et al., 1995）．しかし，PCBによる血中T_4濃度の低下と肝臓でのT_4-UDP-GT活性の増加には必ずしも定量的関連性は見られない（Craft et al., 2002; Kato et al., 2003a）など不明な点もある．また，PCBの血中甲状腺ホルモンへの影響に関する研究は，ほとんどラットを用いて行なわれたものであり，その低下作用における動物種差については，ほとんど知られられていない．

そこで，著者らはPCB投与による血中T_4濃度低下における動物種差やその発現機序を明らかにする目的で，まずはじめに，種々動物種におけるPCBの血中T_4濃度への影響を検討し，次いで各動物におけるPCB代謝やT_4-UDP-GT誘導性の相違を追求した．本稿では，これまでに得られた成果を紹介する．

1．PCBの血中甲状腺ホルモン量への影響：動物種差

種々の用量のKanechlor-500（KC500，PCB製品であるPCB混合物），2,2',4',5,5'-pentachlorobiphenyl (PentaCB) あるいは2,2',3',4',5,6-hexachlorobiphenyl (HexaCB) をマウスに投与し，血中total T_4濃度を50%低下させる各PCBの用量を求めた．次いで，各PCBを本用量でddY系マウス，Syrian系ハムスター，Wistar系ラットあるいはHartley系モルモットに処理し，血中甲状腺ホルモン濃度への影響を調べた．

その結果，マウス，ハムスター，ラットおよびモルモットに，KC500を投与すると，用いたいずれの動物種でも血中total T_4およびfree T_4濃度の低下が認められた

Table 3 Hepatic concentrations of $MeSO_2$-PCB metabolites after the administration of KC500, PentaCB or HexaCB to mice, hamsters, rats and guinea pigs

Treatment	Animals	Total $MeSO_2$-PCBs (ng/g liver)
KC500	Mice	267
	Hamsters	46
	Rats	102
	Guinea pigs	138
PentaCB	Mice	265
	Hamsters	13
	Rats	62
	Guinea pigs	31
HexaCB	Mice	577
	Hamsters	31
	Rats	190
	Guinea pigs	270

Animals were killed at 4 days after the administration of KC500 (37.5 mg/kg, ip), PentaCB (11 mg/kg, ip) or HexaCB (19 mg/kg, ip). Results are expressed as the mean for 5-6 animals.

(Table 2). 一方, total T_3濃度の低下はモルモットのみであった.

また, PentaCB投与では, マウス, ハムスターおよびラットで, HexaCB投与では, マウスのみに血中total T_4濃度の低下が見られた. なお, マウスにPentaCBを投与したときのみtotal T_3濃度の低下も見られた.

血中甲状腺刺激ホルモン (TSH) 濃度は4種の動物にいずれのPCBを投与した場合にも変化しなかった (Table 2).

これらの結果から, PCB投与による血中甲状腺ホルモン濃度への影響には, 少なくとも一部, 動物種差が見られることが明らかになった (Kato et al., 2003b; 2003c).

血中total T_4濃度の低下 (あるいはその動物種差) を生む要因として, ① 甲状腺への直接作用 (および各動物の甲状腺のPCBあるいはその代謝物に対する感受性の相違) や, ② PCBやその代謝物によるT_4-UDP-GTの誘導 (およびその動物種差), また, ③ PCBやその代謝物の血中T_4輸送タンパク (トランスサイレチン : TTR) との結合などが考えられる.

以下, 血中T_4濃度の低下機構について, 動物種差発現の解明を目指し, 検討した結果を記す.

2. 甲状腺への直接的作用

PCBによる血中T_4濃度の低下の要因として, 甲状腺濾胞上皮細胞におけるT_4生合成の抑制や, 甲状腺からのT_4の放出抑制が考えられる (Collins and Capen, 1980; Saeed and Hansen, 1997). そこで, マウス, ハムスター, ラットあるいはモルモットに, KC500, PentaCBあるいはHexaCBを投与し, 甲状腺の空胞変性, 濾胞上皮細胞の肥大および過形成について検討した.

各PCBを投与したいずれの動物にも甲状腺への上記悪影響は見られず, 各PCB投与時に起こる血中T_4濃度の低下は, PCBあるいはその代謝物による甲状腺への直接作用によるものではないことが示唆された.

3. PCB類の代謝とその動物種差

PCBのメチルスルフォン代謝物の3-メチルスルフォニル- (3-$MeSO_2$-) および4-$MeSO_2$-PentaCB, および3-$MeSO_2$-HexaCBなどは, UGT1A1/6誘導活性を有し, この酵素誘導がT_4の代謝を亢進させ, 血中T_4濃度の低下を引き起こすと考えられている (Kato et al., 2000). したがって, $MeSO_2$代謝物の生成量の違いが, 各動物種における血中T_4濃度の低下に差を生む要因になっている可能性が考えられる.

そこで, マウス, ハムスター, ラットあるいはモルモットにKC500, PentaCBあるいはHexaCBを投与し, 血中T_4濃度の低下と, $MeSO_2$代謝物の生成量との関連性を検討した. KC500を投与後の肝臓中の総$MeSO_2$代謝物量は, マウスで最も高く, モルモット, ラット, ハムスターではそれぞれマウスの約1/2, 1/3, および1/6量であった (Table 3). また, PentaCB投与後の$MeSO_2$代謝物生成量もマウスで最も高く, 続いてラット, モルモット, ハムスターの順であった. さらに, HexaCB投与でも, $MeSO_2$代謝物はマウスにおいて最も多く生成され, その生成量は, 以下, モルモット>ラット>ハムスターの順であった (Table 3).

以上, 各PCB投与時の, 各動物での血中T_4濃度の低下と肝臓中各$MeSO_2$代謝物量との間には必ずしもはっきりとした相関性は認められず, 血清中total T_4濃度の低下が, 単に$MeSO_2$代謝物の生成量に依存して起こるのではないことが示唆された.

4. 肝UDP-GTへの影響

一般に, PCBによるラット血中T_4濃度の低下の主因として, T_4のグルクロン酸抱合をはじめとするT_4の代謝系の促進が考えられている (Barter and Klaassen, 1994; Schuur et al., 1997; van Birgelen et al., 1995). そこで, PCB投与時の各動物における血中T_4濃度の低下と, 肝T_4-UDP-GT活性ならびに胆汁中へのT_4のグルクロン酸抱合体排泄量との関連性を調べた.

モルモットにKC500を, また, マウスにHexaCBを投与すると, いずれの場合にも肝T_4-UDP-GTが誘導された (Table 4). しかし, T_4のグルクロン酸抱合体の胆汁排泄量には有意な変化は見られなかった (Table 4). KC500をマウス, ハムスター, ラットに投与した場合にも, モルモットの場合と同様に, 血中T_4濃度は低下するものの, T_4-UDP-GTの誘導は, モルモットの場合とは異なり, 起こらなかった (Tables 2 and 4). このように, KC500やHexaCBによる血中T_4濃度の低下を, 単に

Table 4 Effects of KC500, PentaCB and HexaCB on hepatic microsomal T_4-UDP-GT activity and the biliary excretion of [^{125}I]T_4-glucuronide in mice, hamsters, rats and guinea pigs

Treatment	Animals	T_4-UDP-GT	[^{125}I]T_4-glucuronide
KC500	Mice	→	→
	Hamsters	→	→
	Rats	→	↑
	Guinea pigs	↑	→
PentaCB	Mice	→	n.t.
	Hamsters	→	n.t.
	Rats	→	n.t.
	Guinea pigs	→	n.t.
HexaCB	Mice	↑	n.t.
	Hamsters	→	n.t.
	Rats	→	n.t.
	Guinea pigs	→	n.t.

Animals were killed at 4 days after the administration of KC500 (37.5 mg/kg, ip), PentaCB (11 mg/kg, ip) or HexaCB (19 mg/kg, ip).
Results are expressed as the mean for 4-6 animals. n.t.: not tested.

UDP-GTの誘導やT_4の胆汁排泄量で説明することは難しい.

また，PentaCB投与の場合には，マウス，ハムスターおよびラットのいずれでもUDP-GTは誘導されず，各動物で見られる血中T_4濃度の低下を，単にUDP-GT誘導で説明することはできなかった．なお，PentaCBを投与したモルモットでは，血中T_4濃度の低下やUDP-GTの誘導は認められなかった（Tables 2 and 4）.

そこで，PCB投与時のラットにおける血中T_4濃度の低下に，肝UDP-GTの誘導（活性上昇）が関与しているか否かをより明確にするために，Wistar系ラットおよびGunnラット（遺伝的にUGT1Aサブファミリーを欠損したWistar系ラットの突然変異体）を用い，PentaCB（112 mg/kg）あるいはKC500（100 mg/kg）投与後の血中T_4濃度の変動と，肝T_4-UDP-GTの発現量およびT_4グルクロン酸抱合活性との関連性を追究した.

血中total T_4，free T_4濃度は各PCB投与により両ラットで著しく低下した（Table 5）．一方，T_4-UDP-GT（UGT1A, UGT1A1, UGT1A6）の発現量およびT_4-UDP-GT活性はWistar系ラットで著しく増加したが，Gunnラットではこれら発現量や活性には有意な変化は認められなかった（Table 6）.

したがって，KC500あるいはPentaCB投与時のGunnラットに見られる血中T_4濃度の低下は，肝T_4-UDP-GTの活性には依存していないことが示された．このことは，Wistar系ラットでのKC500あるいはPentaCB投与による血中T_4濃度の低下もまた，少なくとも一部T_4-UDP-GT非依存的な機序で起こっている可能性を示唆している（Kato et al., 2004）.

Table 5 Effects of KC500 and PentaCB on the levels of serum total T_4 and free T_4 in Wistar and Gunn rats

Treatment	Total T_4 (% of control)		Free T_4 (% of control)	
	Wistar	Gunn	Wistar	Gunn
Control	100	100	100	100
KC500	17	19	15	18
PentaCB	23	17	39	15

Animals were killed at 4 days after the administration of KC500 (100 mg/kg, ip) or PentaCB (112 mg/kg, ip).
Results are expressed as the mean for 3-8 animals.

5. トランスサイレチンとの結合

PCBの水酸化代謝物は血中T_4の輸送タンパクであるTTRと親和性をもち，T_4と競合的に結合することが報告されている（Lans et al., 1993）．この競合的結合が血中T_4の標的器官への輸送を撹乱し，血中T_4濃度を低下させる可能性が考えられる（Brouwer et al., 1998; Meerts et al., 2002）．そこで，マウス，ハムスター，ラット，モルモットにKC500（37.5 mg/kgおよび100 mg/kg）および[^{125}I] T_4を投与し，血中[^{125}I] T_4とTTRあるいはアルブミンとの結合率の変動を測定した.

その結果，ラットおよびモルモットでは，KC500投与によりT_4とTTRとの結合阻害が起こり，T_4とアルブミンとの結合率が増加することが明らかになった．一方，マウスおよびハムスターではPCBを投与しても，T_4と血中タンパク（TTR，アルブミン）との結合率にはほとんど変化は認められなかった.

したがって，ラットとモルモットでのKC500投与による血中T_4濃度の低下には，KC500（PCBs）あるいはそれらの水酸化代謝物によるTTRとの結合が関与している可能性が考えられるが，マウスやハムスターにおける血中T_4濃度の低下には，このような機構は考え難い.

6. 血中T_4の肝臓への移行

前述までの研究結果から，PCBによる血中T_4濃度の低下機序やその動物種差を十分に説明することは，困難であった．そこで，T_4の体内動態に注目して，マウス，ハムスター，ラットあるいはモルモットにKC500を投与した場合の，[^{125}I]T_4のクリアランスをさらに調べた．KC500（100 mg/kg）投与により，いずれの動物においても，血中からの[^{125}I]T_4の血清クリアランスは増し，分布容積（組織移行性）も増加した．また，この組織移行性増加の度合いには，種差が見られ，ラットで4.2倍，ハムスターで3.7倍，モルモットで1.8倍，マウスで1.4倍であった.

これらの結果から，KC500投与による血中T_4濃度の低下には，血中から組織へのT_4の急速な移行が関与し

Table 6 Effects of KC500 and PentaCB on the levels of the hepatic microsomal UGT isoforms and T_4-UDP-GT activity in Wistar and Gunn rats

Treatment	UGT1A (% of control)		UGT1A1 (% of control)		UGT1A6 (% of control)		T_4-UDP-GT (% of control)	
	Wistar	Gunn	Wistar	Gunn	Wistar	Gunn	Wistar	Gunn
Control	100	n.d.	100	n.d.	100	n.d.	100	100
KC500	370	n.d.	138	n.d.	642	n.d.	392	111
PentaCB	163	n.d.	167	n.d.	106	n.d.	202	141

Animals were killed at 4 days after the administration of KC500 (100 mg/kg, ip) or PentaCB (112 mg/kg, ip). Results are expressed as the mean for 4-10 animals. n.d.: not detected.

ていることが示唆された．そこで，血中から消失した[^{125}I]T_4がどのような組織に移行したかを明らかにするために，KC500（100 mg/kg）投与後の，[^{125}I]T_4の組織分布を調べた．まずはじめに，各対照（KC500未処理）動物に[^{125}I]T_4を投与し，その組織分布を測定した．その結果，分布量は肝臓で特に高いことが判明した．また，KC500投与により，いずれの動物においても，特に肝の分布量が顕著に増加することが明らかになった．

したがって，KC500による血中T_4濃度の低下には，血中T_4の速やかな肝臓への移行が関わっているものと考えられる．

7．甲状腺ホルモンのトランスポーター

最近，有機アニオン輸送ポリペプチド（Oatp1，Oatp2，Oatp3，Oatp4）（Abe et al., 1998; Cattori et al., 2000; Friesema et al., 1999），Na^+/タウロコール酸共輸送ペプチド（Ntcp）（Friesema et al., 1999），L型アミノ酸トランスポーター（LAT1，LAT2）（Friesema et al., 2001），モノカルボン酸トランスポーター（MCT8）（Friesema et al., 2003）などが甲状腺ホルモンをも基質とすることが次々と明らかにされている．また，T_4の輸送に関わるとされるOatp1，Oatp2，Oatp3，Oatp4，NtcpおよびMCT8がラットの肝臓に発現していることも報告されている（Friesema et al., 2003; Li et al., 2002; Meier, 1995）．

そこで，ラットにKC500を投与し，各トランスポーター遺伝子発現への影響をRT-PCR法を用いて測定した．KC500をラットに投与すると，肝臓のOatp2 mRNAおよびLAT1 mRNAの発現量が有意に増加した．これらの結果は，これらトランスポーターの発現増加が肝臓のT_4蓄積量の増加，そして，血中T_4濃度の低下をもたらす要因になっている可能性を示している．

結　　語

マウス，ハムスター，ラットあるいはモルモットに，KC500を投与した場合，いずれの動物でも血中total T_4濃度の低下が見られた．また，マウス，ハムスター，ラットにPentaCBを投与した場合や，マウスにHexaCBを投与した場合にも，血中total T_4濃度の低下が見られたが，これら投与時には，一部，種差が認められた．

本研究では，各PCB投与による各動物の血中T_4濃度の低下機序やその動物種差を明らかにするため，PCB投与時の甲状腺の病理組織学的検討をはじめ，肝臓T_4-UDP-GTの誘導（活性増加），PCBの代謝，血中T_4-TTR結合量の変化，および肝臓でのT_4トランスポーター発現量への影響などを検討した．しかしながら，PCB投与による血中T_4濃度の低下機序やその動物種差を，単一の要因で説明することは難しく，これら検討した要因，あるいは未知の要因が複雑に絡み合って各動物の感受性が決定されているように考えられる．これまで，PCBによる血中T_4濃度の低下は，肝臓のT_4-UDP-GTの誘導（活性増加）が主因とされてきたが，本研究結果は，本酵素誘導は，単に一つの要因であるに過ぎないことを示唆し，PCBによる毒性発現は極めて複雑な機序を通して起こっていることを示している．

以上，ヒトを含む多くの動物種のPCBによる血中甲状腺ホルモン濃度の低下機構の解明はいまだ不十分であり，今後のさらなる研究が必要である．

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目次

第3号

巻頭言

「油症」を診て感じたこと

(九州大学大学院皮膚科学教授)
古江増隆

油症の患者さんをはじめて診察したのは、九州大学に着任した翌年の1998年のことです。多くの患者さんは昔に比べれば症状はかなりよくなったとおっしゃっていましたが、耳周囲、顔、うなじ、腋窩、陰部などに多発した面疱や嚢腫を診たときは驚きました。皮膚科医ですので、皮膚症状そのものにびっくりしたわけではありません。油症が発生して30年も経っているのに、強い症状に悩んでおられる患者さんがいまだにいらっしゃるのがショックだったのです。「今でも時々膿むのが悲しい」という老婦人の言葉にも心が痛みました。

2000年に九州大学油症治療研究班長を、2001年から全国油症治療研究班長を拝命しました。正直に申し上げますと、いったい何をすればいいのか不安でたまりませんでした。でも患者さんたちのことを考えると愚痴などこぼせないと気を引き締め、油症の文献をはじめから読むことにしました。油症研究班の業績は発生当時から克明に記載され発表されており、また倉恒匡徳先生他監修による英文単行本「Yusho」も発刊されていました。油症はPCBだけでなくPCDFなどのダイオキシン類によって汚染されたカネミ油の摂取による食中毒です。患者さんの血中PCB濃度やパターンは測定されていましたが、ダイオキシン類の血中濃度測定は測定感度や再現性の問題から検診では行なわれておりませんでした。一方、研究班内ではダイオキシン類をかなり低コストで高精度に測定することが可能になりつつありました。そこでダイオキシン類の血中濃度測定を検診に組み込むことは、医学的根拠に基づく診断基準の改訂につながるだけでなく、認定者の病態をより詳細に把握することが可能となり、ひいてはダイオキシン類の濃度を下げる薬剤の開発や症状軽減の治療薬の開発につながるという共通の考えが班内に自然と広がりました。厚労省の担当課の方々も即座に賛同いただき全面的にご援助くださり、感度・再現性・妥当性をクリアし、2004年9月29日にPCDF濃度を加えた新診断基準が作成されました。PCB/ダイオキシン類濃度と様々な症状や検査値がどのような関係にあるのか、なんらかの治療薬を開発できないかなどを今後のフォローの中心課題にすえたいと考えています。油症に関する情報は以下のサイトをご覧ください。「油症の検診と治療の手引き2004」；http://www.kyudai-derm.org/ yusho/index.html、「油症研究30年の歩み・小栗一太他監修」；http://www.kyudai-derm.org/yusho_kenkyu/ index.html

Freshwater crocodile
ワニにも影響か？

Saltwater crocodile

Polychlorinated biphenylsによる血中サイロキシン濃度低下作用機構

静岡県立大学薬学部　加藤善久、山田静雄、出川雅邦

Polychlorinated biphenyl (PCB)は、多くの野生生物の組織のみならず、ヒトの血液、母乳、肝臓、脂肪組織などにも見出され、生体に対する影響が懸念されている。ラットでは、すでに、PCB投与により血中サイロキシン(T_4)濃度が低下することが報告され、ヒトにおいてもPCB曝露により甲状腺ホルモン（T_4、3,5,3'-トリヨードサイロニン(T_3)）の撹乱が引き起こされる可能性が指摘されている。一般に、PCBによるラットでの血中T_4濃度の低下は、肝臓のUDP-グルクロン酸転移酵素(UDP-GT)が誘導され、T_4のグルクロン酸抱合化や胆汁排泄が促進されることによると考えられている。しかし、PCBによる血中T_4濃度の低下と肝臓でのT_4-UDP-GT活性の増加には、必ずしも定量的関連性は見られないなど、このT_4の低下メカニズムについては、不明な点が多く残されている。

そこで、PCB投与時のラットにおける血中T_4濃度の低下に、肝UDP-GTの誘導が関与しているか否かをより明確にするために、Wistar系ラットおよびGunnラット（遺伝的にUGT1Aサブファミリーを欠損したWistar系ラットの突然変異体）を用い、2,2',4',5,5'-pentachlorobiphenyl (PentaCB)あるいはKanechlor-500 (KC500、PCB製品であるPCB混合物)投与後の血中T_4濃度と、肝T_4-UGT-GT (UGT1A、UGT1A1、UGT1A6)の発現量およびT_4のグルクロン酸抱合活性との関連性を検討した。

その結果、血中total T_4、free T_4濃度は、各PCB投与によりWistarとGunnの両ラットともに著しく低下することが明らかになった。一方、Wistar系ラットにおいては、UGT1A、UGT1A1、UGT1A6の発現量およびT_4-UDP-GT活性が著しく増加したが、Gunnラットではこれら発現量や活性には有意な変化は認められなかった。したがって、KC500あるいはPentaCB投与時のGunnラットに見られる血中T_4濃度の低下は、肝T_4-UDP-GTの活性には依存していないことが示された。このことは、Wistar系ラットでのKC500あるいはPentaCB投与による血中T_4濃度の低下もまた、少なくとも一部T_4-UDP-GT非依存的な機序で起こっている可能性を示唆している。

また、T_4のグルクロン酸抱合のほかに、T_4の代謝を亢進させる生体内反応として、脱ヨード化反応が知られている。そこで、KC500およびPentaCBを投与したWistarおよびGunnラットの肝のI型ヨードサイロニン脱ヨード化酵素活性を測定した。しかし、いずれのラットでも、本酵素活性の増加は認められなかった。なお、Aroclor1254を用いた場合にも、同様の結果が報告されている。したがって、WistarおよびGunnラットにPCBを投与した場合の血中T_4濃度の低下には、I型ヨードサイロニン脱ヨード化酵素はほとんど関与していないと考えられる。

PCBの水酸化代謝物は、血中T_4の輸送タンパクであるトランスサイレチン（TTR）と親和性をもち、T_4と競合的に結合すること、さらに、TTRとの親和性はモノヒドロキシ体よりジヒドロキシ体の方が高いことが報告されている。したがって、これらの競合的結合が、血中T_4の標的器官への輸送を撹乱し、血中T_4濃度を低下させる可能性も考えられる。

今回、KC500を投与したWistarとGunnラットにおける主水酸化代謝物は4-OH-2,3,3',4',5-pentachlorobiphenyl（T_4の3.3倍の結合親和性を持つ）であり、それらの割合は、それぞれ血中全水酸化体の89％、56％であった。なお、Gunnラットでは、37％がジヒドロキシ体であった。また、PentaCBを投与したWistarやGunnラットの血中全水酸化体濃度の80％以上は3',4'-$(OH)_2$-PentaCBであった。

このことは、ラットでのKC500あるいはPentaCB投与による血中T_4濃度の低下の一部は、PCBの水酸化代謝によって引き起こされる可能性を示唆している。

以上、PCBを投与した動物における血中T_4濃度の低下機序やその動物種差を明らかにするため、著者らは、すでに、PCB投与時の甲状腺の病理組織学的解析をはじめ、肝臓でのT_4-UDP-GTの誘導、PCBの代謝および肝臓でのT_4トランスポーター発現量などへの影響を検討してきた。しかしながら、PCB投与による血中T_4濃度の低下やその動物種差を、単一の要因で説明することは難しく、これまでに検討してきた要因、あるいは未知の要因が複雑に絡み合って、血中T_4濃度の低下が惹起されるものと考えられる。

これまで、PCBによる血中T_4濃度の低下は、主に肝臓のT_4-UDP-GTの誘導が主因とされてきたが、本研究結果は、本酵素誘導は、単にひとつの要因であるに過ぎないことを明らかにし、PCBによる生体影響が極めて複雑な機序で現れてくることを改めて示している。このように、PCBによる血中甲状腺ホルモン濃度の低下機構の解明はいまだ不十分であり、今後のさらなる研究が必要である。

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Modulation of oestrogen receptor signalling by association with the activated dioxin receptor

Fumiaki Ohtake[*1], Ken-ichi Takeyama[*1,2], Takahiro Matsumoto[*1], Hirochika Kitagawa[*1], Yasuji Yamamoto[*3], Keiko Nohara[*4,5], Chiharu Tohyama[*4,5], Andree Krust[*6], Junsei Mimura[*5,7], Pierre Chambon[*6], Junn Yanagisawa[*1,2], Yoshiaki Fujii-Kuriyama[*5,7] & Shigeaki Kato[*1,2]

[*1] *The Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-0032, Japan*
[*2] *SORST, Japan Science and Technology, Kawaguchi, Saitama 332-0012, Japan*
[*3] *Taiho Pharmaceutical Company Ltd, Cancer Research Laboratory, Hanno Research Center, Hanno, Saitama, 357-8527, Japan*
[*4] *National Institute for Environmental Studies, Tsukuba, Ibaraki 305-8506, Japan*
[*5] *CREST, Japan Science and Technology, Kawaguchi, Saitama 332-0012, Japan*
[*6] *Institut de Génétique et de Biologie Moleculaire et Cellulaire, CNRS, INSERM, Université Louis Pasteur, Collège de France, 67404 Illkirch, Strasbourg, France*
[*7] *TARA Center, University of Tsukuba, Tennodai, Tsukuba, 305-8577, Japan*

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Modulation of oestrogen receptor signalling by association with the activated dioxin receptor

Fumiaki Ohtake*, Ken-ichi Takeyama*†, Takahiro Matsumoto*, Hirochika Kitagawa*, Yasuji Yamamoto‡, Keiko Nohara§‖, Chiharu Tohyama§‖, Andree Krust¶, Junsei Mimura‖#, Pierre Chambon¶, Junn Yanagisawa*†, Yoshiaki Fujii-Kuriyama‖# & Shigeaki Kato*†

** The Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-0032, Japan*
† SORST, Japan Science and Technology, Kawaguchi, Saitama 332-0012, Japan
‡ Taiho Pharmaceutical Company Ltd, Cancer Research Laboratory, Hanno Research Center, Hanno, Saitama, 357-8527, Japan
§ National Institute for Environmental Studies, Tsukuba, Ibaraki 305-8506, Japan
‖ CREST, Japan Science and Technology, Kawaguchi, Saitama 332-0012, Japan
¶ Institut de Génétique et de Biologie Moleculaire et Cellulaire, CNRS, INSERM, Université Louis Pasteur, Collège de France, 67404 Illkirch, Strasbourg, France
 # TARA Center, University of Tsukuba, Tennodai, Tsukuba, 305-8577, Japan

Environmental contaminants affect a wide variety of biological events in many species. Dioxins are typical environmental contaminants that exert adverse oestrogen-related effects[1]. Although their anti-oestrogenic actions[2,3] are well described, dioxins can also induce endometriosis[4–7] and oestrogen-dependent tumours[8,9], implying possible oestrogenic effects. However, the molecular mechanism underlying oestrogen-related actions of dioxins remains largely unknown. A heterodimer of the dioxin receptor (AhR) and Arnt, which are basic helix–loop–helix/PAS-family transcription factors, mediates most of the toxic effects of dioxins[10,11]. Here we show that the agonist-activated AhR/Arnt heterodimer directly associates with oestrogen receptors ER-α and ER-β. This association results in the recruitment of unliganded ER and the co-activator p300 to oestrogen-responsive gene promoters, leading to activation of transcription and oestrogenic effects. The function of liganded ER is attenuated. Oestrogenic actions of AhR agonists were detected in wild-type ovariectomized mouse uteri, but were absent in AhR$^{-/-}$ or ER-α$^{-/-}$ ovariectomized mice. Our findings suggest a novel mechanism by which ER-mediated oestrogen signalling is modulated by a co-regulatory-like function of activated AhR/Arnt, giving rise to adverse oestrogen-related actions of dioxin-type environmental contaminants.

ERs, which are members of the nuclear receptor (NR) family[12,13], and AhR/Arnt are both ligand-dependent transcription factors. Ligand-activated AhR heterodimerizes with Arnt and activates the transcription of dioxin target genes such as *CYP1A1* (refs 10,11) through xenobiotic response elements (XREs). ERs bind to oestrogen response elements (EREs) and activate transcription in an oestrogen-dependent manner. This transcriptional activation requires the recruitment of co-activator complexes[13–18], including histone acetyltransferase (HAT) complexes containing p300 and CREB binding protein (CBP). In view of previous reports that AhR ligands exhibit oestrogen-related adverse effects, it is possible that ER-mediated oestrogen signalling might cross-talk with AhR-

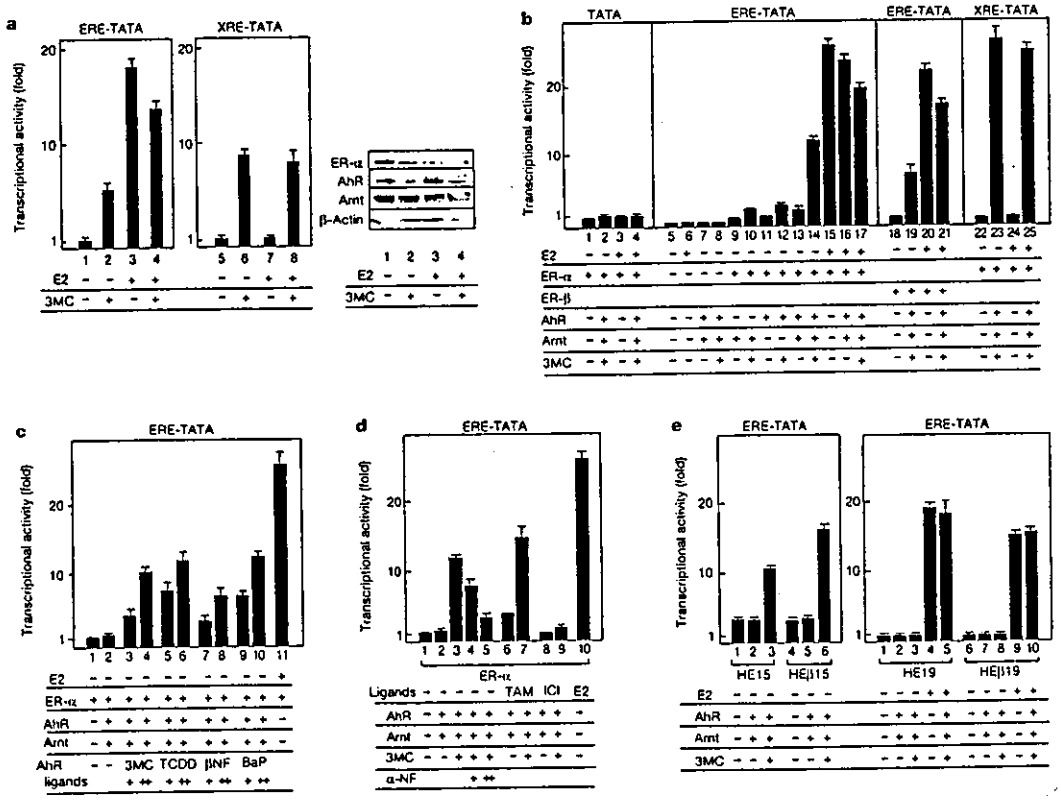

Figure 1 Activation of unliganded ER function by liganded dioxin receptor heterodimer. **a**, A dioxin receptor ligand activates transcription mediated through an ERE. MCF-7 cells were transfected with the reporter plasmids ERE-luciferase or XRE-luciferase in the presence or absence of E2 (10 nM) and 3MC (1 μM). Luciferase assays were performed with the cell extracts. All values are means ± s.d. for at least three independent experiments. **b**, Liganded AhR/Arnt induces the transactivation function of ERE-bound unliganded ER. Ishikawa cells transfected with the indicated plasmids were subjected to luciferase assays. **c**, Transactivation of unliganded ER by the other AhR agonists. **d**, Potentiation of ERE-mediated transcription by liganded AhR/Arnt is blocked by an antagonist for either ER-α or AhR. Cells treated with tamoxifen (TAM; 100 nM), ICI182,780 (ICI; 100 nM), 3MC (+, 100 nM; ++, 1 μM), TCDD (+, 10 nM; ++, 100 nM), β-naphthoflavone (β-NF; +, 100 nM; ++, 1 μM), benzo[a]pyrene (BaP; +, 10 nM; ++, 100 nM), α-naphthoflavone (α-NF; +, 100 nM; ++, 1 μM). **e**, Potentiation of ERE-mediated transcription by AhR/Arnt is mediated by the ERs A/B regions.

mediated signalling through an unknown mechanism that regulates transcription. We therefore decided to examine whether AhR/Arnt heterodimer could transcriptionally affect ER transactivation functions, thereby modulating oestrogen signalling.

To monitor the transactivation function of endogenous receptors, luciferase reporter plasmids bearing consensus binding elements—ERE for ERs, and XRE for AhR/Arnt—were transfected into MCF-7 cells, a breast cancer cell line known to express both receptors endogenously[2]. Although the synthetic AhR ligand 3-methylcholanthrene (3MC) effectively activated transcription through XRE[10], 17β-estradiol (E2) did not, as expected (Fig. 1a). However, to our surprise, 3MC alone activated ERE-mediated transcription in the absence of E2 (Fig. 1a). In the presence of E2, ERE-mediated transcription was decreased by the addition of 3MC. Western blotting showed that the amount of ligand-induced transactivation did not simply reflect variations in receptor numbers (Fig. 1a). 3MC alone decreased AhR and ER-α protein levels, in agreement with previous reports[19].

We then examined the effect of AhR/Arnt on ER-mediated transcription by using exogenous receptors in Ishikawa cells, a uterine tumour cell line. Again, 3MC potently stimulated ERE-mediated transcription in the absence of E2 when both ER (either ER-α or ER-β) and AhR/Arnt were expressed, whereas it lowered the E2-induced transactivation function of ERs (Fig. 1b) without binding directly to ERs (Supplementary Fig. 1a) or affecting expression levels of ERs (data not shown). This activation effect of 3MC requires ERE (Fig. 1b, lanes 1–4), ER-α (lanes 7 and 8), AhR (lanes 9 and 10) and Arnt (lanes 11 and 12). To verify that an AhR ligand does indeed exert oestrogenic action through direct binding to AhR, other AhR ligands were further tested. More stable ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]-pyrene and β-naphthoflavone acted as agonists, like 3MC (Fig. 1c), whereas the oestrogenic action of 3MC was blocked by either a known AhR antagonist, α-naphthoflavone or a pure oestrogen antagonist, ICI182,780 (Fig. 1d). The modulation of transcription activity by AhR/Arnt observed with ERs was not detected on other NRs including glucocorticoid receptor, progesterone receptor, vitamin D receptor (VDR), retinoic acid receptor and peroxisome proliferator activated receptor-γ (PPAR-γ) (data not shown).

Because ERs possess two transactivation functions, AF-1 and AF-2, in the amino-terminal A/B and carboxy-terminal E/F regions, respectively[16,20], we examined the functional association of AhR/Arnt with these two regions using ER deletion mutants (HE15 for

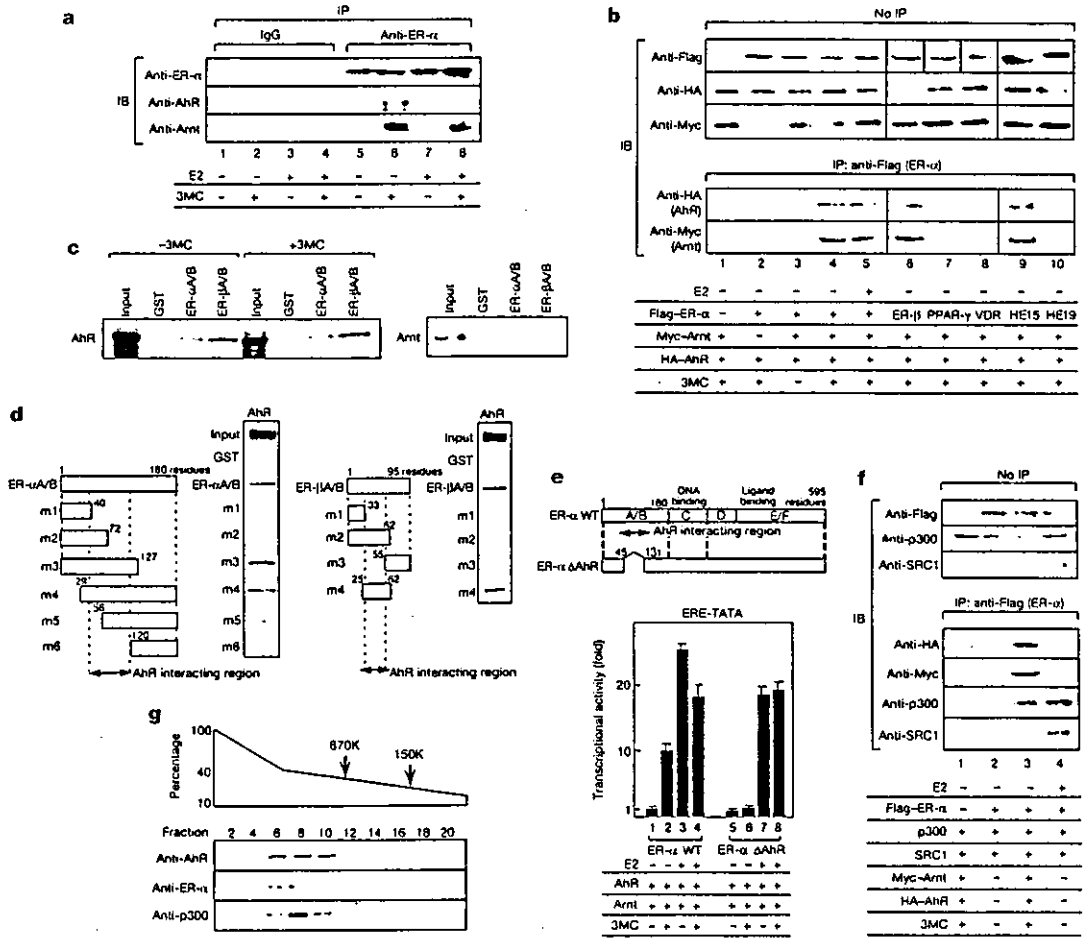

Figure 2 3MC-dependent interaction of ERs with AhR/Arnt. **a**, 3MC-dependent but E2-independent interaction of endogenous ER-α with AhR/Arnt in MCF-7 cells. Cells were subjected to immunoprecipitation (IP) with mouse anti-ER-α or normal mouse immunoglobulin as a control. The immunoprecipitates were western blotted (IB) with specific antibodies as indicated. **b**, E2-independent, 3MC-dependent interaction of exogenous ERs with AhR/Arnt in COS-1 cells. The transfected cells were subjected to immunoprecipitation and then western blotting. PPAR, peroxisomeproliferatoractivated receptor; VDR, vitamin D receptor. **c**, Direct but 3MC-independent interaction of AhR with ER-α and ER-β in an *in vitro* GST pull-down assay. **d**, Mapping the interaction domains of ER-α and ER-β with AhR. **e**, The AhR-interacting core region in the ER-α A/B domain is required for ER-α activation by AhR/Arnt. Luciferase assays with the indicated ER derivative. **f**, Recruitment of p300 co-activator to a complex containing unliganded ER-α and 3MC-bound AhR/Arnt. **g**, AhR/ER–α/p300 form a complex on glycerol gradient analysis. The Flag–AhR associated proteins in stable transformant HeLa cells were fractionated by molecular mass by a glycerol gradient assay.

AF-1 domain, and HE19 for AF-2 domain) (Supplementary Fig. 1b) in Ishikawa cells. The N-terminal A/B regions of ER-α and ER-β were required for stimulation of ERE-mediated transcription by AhR/Arnt, whereas we detected no modulation of AF-2 functions (Fig. 1e)[20]. Thus, 3MC-bound AhR/Arnt might modulate the functions of ERs through association with the N-terminal A/B regions. This possibility was supported by the observation that 3MC-bound AhR/Arnt potentiates the transactivation function of ER-α in the presence of the ER-α AF-1 agonist/AF-2 antagonist tamoxifen (Fig. 1d)[16].

We then tested whether a 3MC-dependent physical interaction occurred between AhR/Arnt and ERs. Irrespective of E2 binding, endogenous ER-α in MCF-7 cells, and tagged ER-α overexpressed in COS-1 cells, were found to co-immunoprecipitate with 3MC-bound AhR, but not with unliganded AhR, only when Arnt was co-expressed (Fig. 2a and b). In agreement with the functional interaction between AhR/Arnt and the A/B region of ER-α (Fig. 1e), a 3MC-dependent interaction between AhR/Arnt and HE15 was observed, but not between AhR/Arnt and HE19 (ref. 12). Although ER-β, like ER-α, also associated with AhR in a 3MC-dependent fashion, no other receptors tested showed such an association (Fig. 2b).

Moreover, a direct interaction between AhR, but not Arnt, and A/B regions from both ER-α and ER-β could be mapped by an *in vitro* glutathione *S*-transferase (GST) pull-down assay (Fig. 2c). It therefore seems that, upon ligand binding and nuclear translocation[16], AhR heterodimerizes with nuclear Arnt and then associates with unliganded ER-α or ER-β, which are constitutively in the nucleus[16], through direct interaction with their A/B regions. Further analyses by GST pull-down assay mapped the small regions of the A/B region of ER-α (residues 40–120), the A/B region of ER-β (residues 33–55)[21], and the helix–loop–helix/PAS domain of AhR[22], which are indispensable for direct interaction *in vitro* (Fig. 2d and Supplementary Fig. 2a). An ER-α mutant lacking the AhR-interacting region (ER-α ΔAhR) failed to be activated by AhR/Arnt but responsiveness to E2 was still retained, supporting the idea that the interaction is required for AhR ligand-induced activation of the ER function (Fig. 2e).

To explore the molecular mechanisms of the 3MC-dependent transactivation function of AhR and ERs, we used co-immunoprecipitation to examine whether p300 was recruited to the complex, because both AhR and ERs have been independently reported to require p300/CRB as a co-activator[10,16,18,23]. p300 was recruited to ER-α in the presence but not the absence of E2 (Fig. 2f, lanes 2 and 4). However, even in the absence of E2, p300 associated with 3MC-bound AhR/Arnt and unliganded ER-α to form a complex (Fig. 2f, lane 3). Recruitment of the p160 family co-activator SRC-1 (ref. 13; Fig. 2f, lane 3), TIF2 or AIB1 (data not shown) to AhR/Arnt-associated ERs were not detected. Thus, the co-activator complex required to activate transcription by the unliganded ERs associated with liganded AhR/Arnt might be distinct from both co-activator complexes for the unassociated receptors. Indeed, ER-α and p300 were detected in the same fractions as Flag ([EYKEEEK]$_2$)-tagged AhR fractionated by a glycerol gradient, suggesting that they form a complex with a relative molecular mass (M_r) larger than 670,000 (670K) (Fig. 2g).

To investigate whether the observed association between AhR and ERs occurred on EREs in endogenous target gene promoters of MCF-7 cells, we performed a chromatin immunoprecipitation (ChIP) analysis with *pS2* and c-*fos* gene promoters[17]. Interestingly, 3MC induced binding of ER-α to ERE, as did E2, with AhR/Arnt recruitment. As expected, 3MC induced the recruitment of AhR/Arnt, but not ER-α, to the *CYP1A1* promoter XRE (Fig. 3a). Reflecting the recruitment of the receptors, acetylation of histone H4 was observed in the promoters (Fig. 3a), indicating the possible recruitment of a HAT co-activator complex to the receptors. The expression of these genes was accordingly induced by 3MC or E2 (Fig. 3d). Thus, the 3MC-dependent association between AhR/Arnt and ER-α seems to promote the binding of unliganded ER-α to EREs.

A ChIP assay involving sequential immunoprecipitation confirmed the 3MC-dependent association of AhR/Arnt with ER-α on

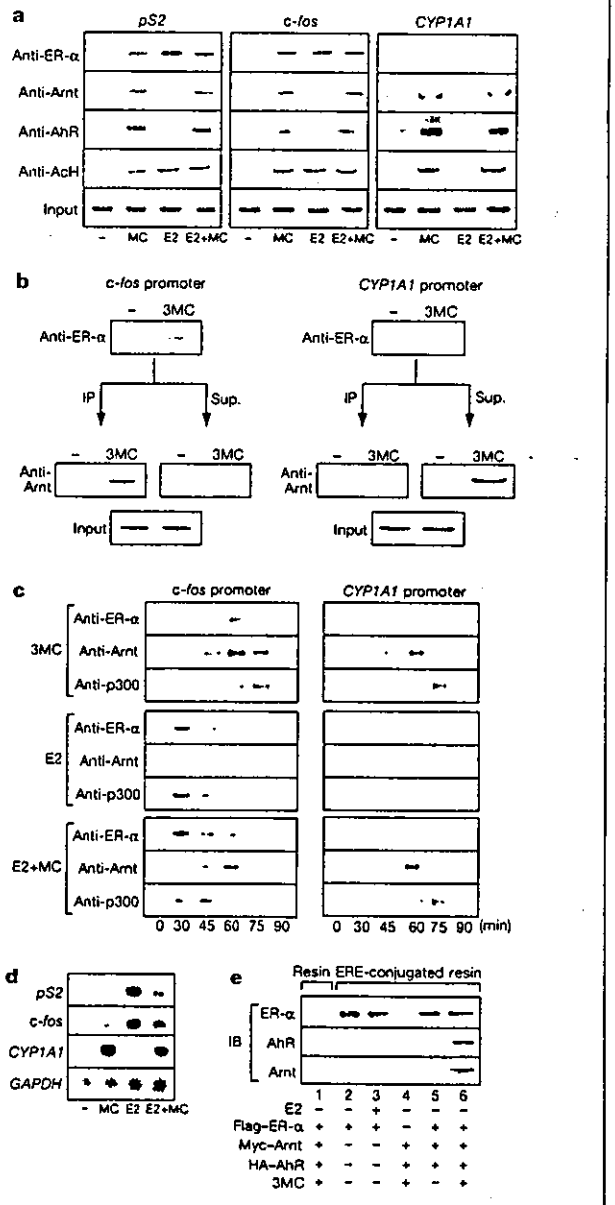

Figure 3 3MC-dependent recruitment of AhR/Arnt to ER-α bound on oestrogen-responsive gene promoters. **a**, 3MC-dependent interaction with AhR/Arnt induces ERE binding of unliganded ER-α to E2 responsive gene promoters in MCF-7 cells. For ChIP analyses, soluble chromatin prepared from MCF-7 cells treated with ligands for 45 min was immunoprecipitated with the indicated antibodies. The final DNA extracts were amplified using specific sets of primer pairs to detect the c-*fos*, *pS2* and *CYP1A1* gene promoters as indicated. **b**, 3MC-dependent association of AhR/Arnt with ER-α bound to E2-responsive gene promoters. The immunoprecipitates and their supernatants were sequentially applied for ChIP analysis as indicated. **c**, Dynamics of ER-α–Arnt–p300 assembly on ligand-responsive gene promoters. Occupancy of the c-*fos* and *CYP1A1* promoters by ER-α, Arnt and p300 at different times after ligand treatments. **d**, Induction of target genes examined by northern blot analysis. **e**, Complex formation of AhR–Arnt–ER-α on ERE through ER-α as revealed by ABCD assay.

ERE (Fig. 3b). A time-course ChIP assay showed that ER-α, AhR and p300 HAT were simultaneously recruited to the c-*fos* promoter, presumably upon the binding of 3MC to AhR (Fig. 3c).

To verify the interaction of AhR/Arnt with ER-α bound to ERE in the promoters, the formation of a complex with ERE was tested by avidin–biotin-conjugated DNA(ERE) (ABCD) precipitation[24] (Fig. 3e). ER-α bound to consensus ERE (Fig. 3e, lanes 2, 3, 5 and 6), whereas AhR/Arnt alone did not (Fig. 3e, lane 4). However, in the presence of ER-α, AhR/Arnt was recruited to ERE in a 3MC-dependent manner (Fig. 3e, lanes 5 and 6). In the transient luciferase assay, the binding of ER-α to ERE and the activation function of both AhR and Arnt were required for the activation of ER-α through ERE by AhR/Arnt (Supplementary Fig. 3a, lanes 3, 7, and 8), whereas the AF-1 and AF-2 activities of ER-α and the DNA-binding capacity of the AhR/Arnt heterodimer were dispensable (Supplementary Fig. 3a, lanes 4–6).

Finally, we tested whether AhR-ligand-dependent AhR–ER interaction was responsible for the oestrogenic actions of AhR agonists in the absence of oestrogens on gene expression in intact animals. In addition to the induction of the *CYP1A1* gene, treatments with 3MC (Fig. 4a) and TCDD (Fig. 4b) for 3 hours stimulated the expression of the oestrogen-responsive genes c-*fos*[25] and *vascular endothelial growth factor* (*VEGF*)[26] in the uteri of ovariectomized wild-type mice (Fig. 4a, b). This oestrogenic action of 3MC in the uterus was also detected in intact 21-day-old female mice, whereas the AhR agonists exhibited anti-oestrogenic activities in the presence of high doses of oestrogen (Fig. 4a). There have been conflicting reports on the induction of c-*fos* by AhR ligands: one is that AhR ligands repress the E2-induced expression of c-*fos*[3]; the other is that AhR ligands themselves induce the expression of c-*fos*[27]. The 3MC-mediated activation of oestrogen target genes was completely abolished in both $AhR^{-/-}$ (ref. 28) and ER-$\alpha^{-/-}$ (ref. 29) ovariectomized mice, although each receptor knockout mouse strain retained ligand responsiveness (Fig. 4a) and the expression (Supplementary Fig. 4a) of the other intact receptor. The injection of 3MC led to increases in uterine wet weight, as did that of E2 (Fig. 4c). This action of 3MC was again abolished in both $AhR^{-/-}$ and ER-$\alpha^{-/-}$ mice (Fig. 4c).

To examine whether the increased uterine wet weight was due to the proliferation of endometrial cells, DNA synthesis in uterine epithelial cells was examined by labelling with bromodeoxyuridine (BrdU). Ovariectomized mice treated with 3MC exhibited enhanced cell proliferation in the glandular epithelium, as did E2-treated mice (Fig. 4d). Proliferation of the luminal epithelium was enhanced by E2 but not by 3MC.

The present findings indicate that the oestrogenic action of AhR agonists might be exerted through a direct interaction between AhR/Arnt and unliganded ER and by the formation of functional units bound to EREs that activate transcription, at least in uterine gene induction and cellular proliferation. The most marked manifestation of the possible oestrogenicity of dioxins could be seen as their linking to endometriosis[4–7], because oestrogen is the major factor in the stimulation of proliferation of these cells. Thus, AhR expressed in the uterine glandular epithelium[30] might respond to dioxins by associating with unliganded ERs, which then stimulates oestrogen-dependent cell proliferation. In contrast, AhR agonists exhibit anti-oestrogenic activities in the presence of high doses of E2 in animals[3] and cultured cell lines[2]. We also found that AhR/Arnt repressed E2-bound ER function, which is consistent with these previous reports. However, whereas most previous studies have not examined or mentioned the effects of AhR ligands in the absence of E2, we addressed this issue carefully in the present study. Thus, oestrogen concentrations, which vary with age, oestrous cycle, tissues and other factors, might define the oestrogenic/anti-oestrogenic actions of the AhR ligands in intact animals. Our present model, in which AhR potentiates unliganded ERs but represses

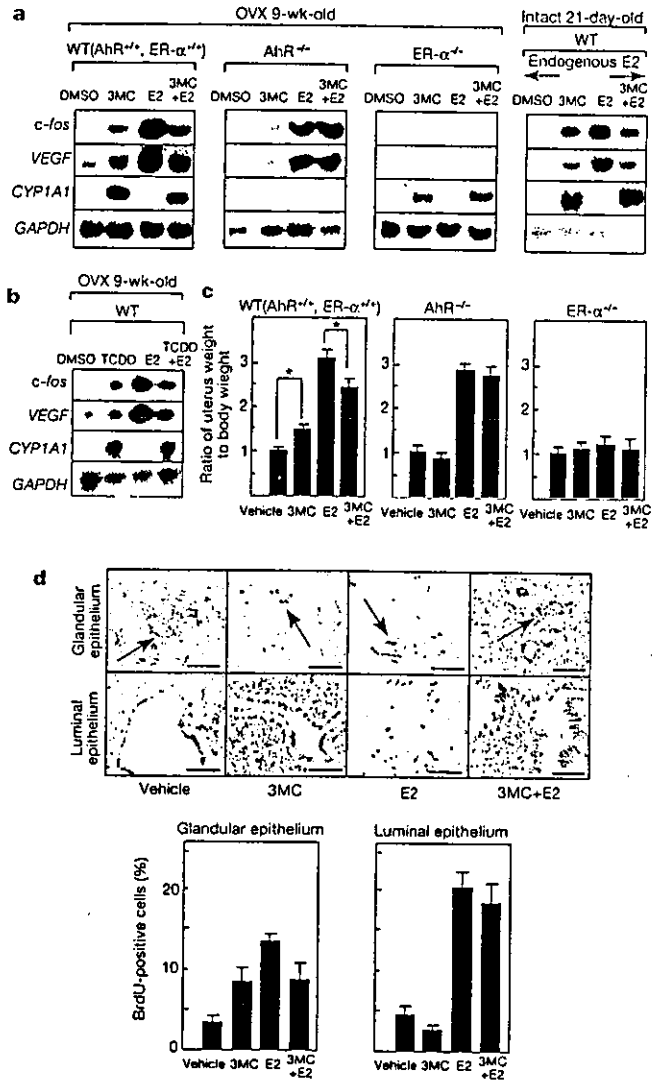

Figure 4 Oestrogenic actions of 3MC in mouse uterus are mediated by AhR and ER-α. **a**, **b**, Induction of E2-responsive genes by AhR agonists is mediated by both AhR and ER-α. Nine-week-old ovariectomized (OVX) mice and intact 21-day-old female mice of the indicated genotypes were injected with the ligands. Three hours later, total RNA was extracted from the uterus, then subjected to northern blot analysis with cDNAs for the target genes for E2 (c-*fos*, *VEGF*) and for 3MC (*CYP1A1*); *GAPDH* cDNA was used as an internal control. WT, wild type. **c**, The 3MC-induced increase in uterine wet weight (measured as the ratio of uterine wet weight in milligrams to body weight in grams) in ovariectomized mice was abolished by inactivation by either AhR or ER-α. The *t*-test shows a significant difference ($P < 0.01$) between 3MC-treated ($n = 9$) and olive-oil-treated ($n = 9$) wild-type mice. There is no significant difference ($P > 0.2$) between 3MC-treated ($n = 4$) and olive-oil-treated ($n = 4$) animals in either $AhR^{-/-}$ and ER-$\alpha^{-/-}$ mice. All values are means ± s.e.m. **d**, Induction of endometrial cell proliferation by 3MC and E2. BrdU-positive cells (brown) are indicated by arrows.

liganded ER, might be an explanation of these previous findings, and it will be of interest to identify the other components of the liganded AhR–ER-α complex involved in the oestrogenic/anti-oestrogenic actions of dioxins. Our proposal is that one of the molecular mechanisms for the oestrogen-related adverse effects of dioxin-type environmental contaminants is the modulation of oestrogen receptor signalling by dioxin-dependent association with dioxin receptor. □

Methods

Plasmids

Full-length complementary DNAs of AhR and Arnt were inserted into pcDNA3 vectors

(Invitrogen). Three consensus EREs[16] and XREs[22] were inserted into the promoter of luciferase pGL3-basic vector to generate ERE-TATA–luciferase and XRE-TATA–luciferase, respectively. ER-α ΔAhR was generated by the deletion of 45–131 residues from ER-α. The other mutants of ER-α and ER-β were as described previously[21].

Transfection and luciferase assay

Human endometrium cancer-derived Ishikawa cells, human breast cancer-derived MCF-7 cells, green monkey COS-1 cells and human 293T cells maintained in DMEM supplemented with 10% FBS were cultured in phenol-red-free DMEM containing 0.2% charcoal-stripped FBS before assays. Cells at 40–50% confluence were transfected with the indicated plasmids (0.25 μg ERE-Luc, 0.1 μg XRE-Luc, 0.025 μg ER-α, AhR and Arnt were transfected) using Lipofectamine reagent (Gibco BRL) in 12-well Petri dishes. Total amounts of cDNA were adjusted by supplementing with empty vector up to 1.0 μg. Cells were treated with E2 (100 nM) and 3MC (1 μM). Luciferase activity was determined with the Luciferase Assay System (Promega)[16]. As a reference plasmid to normalize transfection efficiency, 25 ng pRL-CMV plasmid (Promega) was co-transfected in all experiments. Results are given as means ± s.d. for at least three independent experiments.

Immunoprecipitation and GST pull-down assay

Whole cell extracts[17] were used for immunoprecipitation with either anti-ER-α or anti-Flag antibody (anti-ER-α Ab-4 from Neo Markers; anti-Flag from Santa Cruz Biotechnology) after western blotting with anti-ER-α (Chemicon), anti-Arnt (Santa Cruz Biotechnology), anti-AhR (Santa Cruz Biotechnology), anti-p300 (Upstate Biotechnology), anti-SRC-1 (Santa Cruz Biotechnology), anti-Flag, anti-haemagglutinin and anti-Myc (Invitrogen) antibodies. Normal mouse immunoglobulin was used as a control. For immunoprecipitation of overexpressed proteins, cells were transfected as indicated with Flag-tagged ERs (5 μg), haemagglutinin-tagged AhR (3 μg), Myc-Arnt (5 μg), SRC-1 (0.7 μg) and p300 (0.7 μg) in the presence or absence of 3MC and E2. For the GST pull-down assay, AhR and Arnt were translated *in vitro* and incubated with either GST, GST–ER-α(A/B) or GST–ER-β(A/B) immobilized on glutathione–Sepharose beads[17].

Purification and separation of AhR-interacting complexes

HeLa nuclear extracts were loaded on an M2 anti-Flag agarose gel (Kodak). After being washed with binding buffer, the bound proteins were eluted from the agarose by incubation overnight with 2.5–5.0 ml of the Flag peptide (Kodak) in binding buffer (0.2 mg ml^{-1}). For fractionation on a glycerol gradient, eluents were layered on the top of a 13-ml linear 100–10% glycerol gradient and centrifuged for 16 h at 40,000 r.p.m. in an SW40 rotor (Beckman). Each fraction was western blotted with anti-AhR, anti-ER-α and anti-p300 antibodies. The protein standards used were β-globulin (M_r 158K) and thyroglobulin (667K)[17].

Chromatin immunoprecipitation

Soluble chromatins of MCF-7 cells prepared with the acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology) were immunoprecipitated with antibodies against the indicated proteins. Specific primer pairs were designated to amplify the promoter regions of the *c-fos* (5′-GAAGAGTGGAGAAGGG-3′ and 5′-GAAGCTGTGCTTACGG-3′), *pS2* (5′-AAAGAATTAGCTTAGGCC-3′ and 5′-ACCTTAATCCAGGTCC-3′) or *CYP1A1* (5′-CTTCGCCATCCATTCC-3′ and 5′-GGGACTCCTCTTCGAC-3′) genes from the extracted DNA. Optimal PCR conditions to allow semiquantitative measurement were used on 2% agarose/Tris-acetate-EDTA gels[17]. As a usual condition, cells were treated with ligands for 45 min. The inductions of the target genes were examined by northern blot analysis in MCF-7 cells treated with the ligands for 3 h.

ABCD precipitation

Avidin resin (Promega) was incubated with biotin-conjugated consensus ERE oligonucleotides, followed by incubation with cell lysates in lysis buffer (20 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100 and 1 mM dithiothreitol) for 30 min. The subsequent ERE–protein complexes trapped on the resin were then eluted and western blotted[24].

Oestrogen responses in uterus

Nine-week-old female C57BL/6 mice with the indicated genotypes were ovariectomized. After 2 weeks the mice were treated with 3MC (4 mg kg^{-1}), TCDD (40 μg kg^{-1}), and/or E2 (20 μg kg^{-1}) in olive oil for 3 h. Total RNA was extracted from the uteri by Isogen (Wako Co.) and then subjected to northern blot analysis with cDNAs for the target genes for E2 (*c-fos*, *VEGF*) and for 3MC (*CYP1A1*), with *GAPDH* cDNA (encoding glyceraldehydes-3-phosphate dehydrogenase) as an internal control[16]. For experiments with intact mice, 21-day-old female mice were used.

For uterine weight analysis, mice were treated with ligands for 3 days, and the ratio of uterine wet weight to body weight was calculated, followed by *t*-test analysis. Results are given as means ± s.e.m.

For the BrdU labelling experiment, ovariectomized mice were treated with ligands for 3 days, then injected with BrdU (30 mg kg^{-1}). Paraffin sections from the uteri 8 h after BrdU injection were immunostained with anti-BrdU monoclonal antibody by using the BrdU Labeling and Detection Kit 1 (Roche), and the percentage of BrdU-positive epithelial cells in the sections was calculated.

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Correspondence and request s for materials should be addressed to S.K. (uskato@mail.ecc.u-tokyo.ac.jp).