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Differential Regulation of Thyroid Hormone Receptor-Mediated Function by Endocrine Disruptors

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It is well known that endocrine disruptors (EDs) act as anti-estrogenic agents and affect the function of reproductive organ. EDs are also thought to affect thyroid hormone (TH) system which is important for biological functions such as growth, development and metabolism. However, it is still not clear how EDs are able to regulate TH receptor (TR)-mediated functions. In this study, therefore, the modulatory effects of representative EDs such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyl (Aroclor 1254) and bisphenol A (BPA) were examined using TR-expressing GH_3 cells (a rat pituitary gland epithelial tumor cell line) activated by triiodothyronine (T_3). EDs tested significantly blocked T_3 binding to TR in a dose-dependent manner. Biochemical characterization by Scatchard and Lineweaver-Burk plot analyses indicated that TCDD and aroclor 1254 bound to TH receptors in a competitive inhibitory manner, whereas BPA bound to TH receptors in a non-competitive pattern. The different inhibitory mode of action by EDs was also found in regulating TR-mediated production of prolactin (PRL). Aroclor 1254 exposure for 48 h enhanced T_3-mediated PRL production, but BPA down-regulated. These results suggest that the EDs (TCDD, Aroclor 1254 and BPA) could differentially bind to TR and distinctly regulate the action of TR function, even though EDs are structurally similar.

Key words: Endocrine disrupter, Thyroid hormone, Thyroid hormone receptor, Prolactin

INTRODUCTION

Endocrine disruptors (EDs) cause various abnormalities by blockade of the counteraction of hormones and their receptor, mimic of hormone function, alteration of receptor expression, and diminishment of hormone production (Naciff and Daston, 2004; Stoker *et al.,* 2000). EDs alter natural hormone functions by disrupting hormone binding to their receptors without any cellular response by themselves (Funabashi *et al.,* 2003; Iwamuro *et al.,* 2006). Many endocrine disrupting chemicals are capable of interfering with the proper functioning of estrogen, androgen and thyroid hormones in humans and animals (Naciff and Daston, 2004; Stoker *et al.,* 2000). Exposures to EDs can cause sterility or decreased fertility, impaired development, birth defects of the reproductive tract, and metabolic disorders (Juberg, 2000; Stoker *et al.,* 2000). These chemicals have been shown to alter levels of male and female hormones, as well as certain thyroid hormones. Changes in these hormone levels affect developing organisms and can result in abnormalities in normal physiological responses (such as reproduction, growth, and development), as well as pathological situations (such as cancer and immune system disorders), even at very low levels of exposure (Mizuyachi *et al.,* 2002).

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (Aroclor 1254) (Fig. 1) are a family of EDs with related properties and toxicity. The studies on the effects of dioxin on mice, rats, and hamsters indicate that TCDD causes cancer in the liver, lung, tongue, roof of

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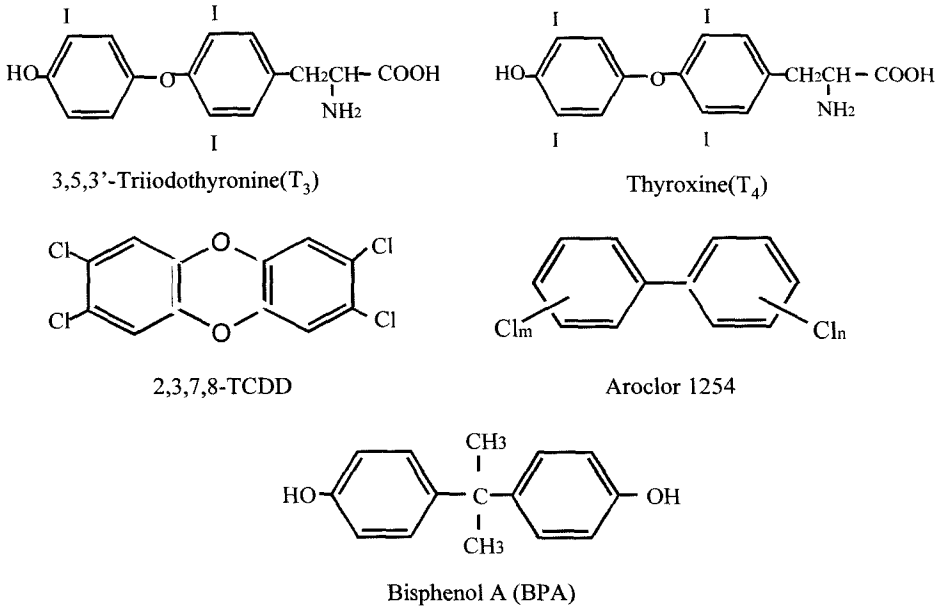

Fig. 1. Chemical structures of thyroid hormones (T_3 and T_4) and EDs

the mouth, nose, thyroid gland, thymus gland, adrenal gland, skin of the face, and under the skin (Hurst *et al.*, 2005). TCDD and Aroclor 1254 cause the activation of carcinogen by induction of cellular CYP1A1 and CYP1A2 through aryl hydrocarbon receptor (Lai *et al.*, 2004; Takahashi *et al.*, 2005). Some PCB molecules have been known to mimic thyroid hormone and interfere with its transport into the developing brain (Porterfield, 1994). Aroclor 1254 metabolites are also known to interfere with neurotransmitter levels in the developing brain. Bisphenol A (BPA) (Fig. 1) is a primarily component used for polycarbonate and epoxy resins. Thus, BPA-based epoxy resins are widely used in consumer products, including composite dental sealants and the inner coating of food cans. Indeed, significant amounts of BPA have been determined in the liquid from canned vegetables and nursing bottle (Brotons *et al.*, 1995). The estrogenic activities of BPA, which include binding to estrogen receptors, induction of progesterone receptors and promotion of cell proliferation in estrogen-responsive MCF-7 breast cancer cells were previously reported (Iso *et al.*, 2006; Singleton *et al.*, 2006).

In spite of that EDs have the structural similarity with thyroid hormone (TH) (Fig. 1), the effects of EDs on TH-mediated biological responses have been recently explored. So far, it has been reported that EDs are acting as strong TH receptor (TR) antagonists (Miyazaki *et al.*, 2004) and in turn affect TH/TR-mediated numerous responses such as the transcriptional regulation of gene clusters involved in brain development. However, how EDs can modulate TH response in terms of TR level was not fully elucidated. In this study, therefore, biochemical feature of the binding pattern of EDs (TCDD, aroclor 1254 and BPA) to TR was carefully examined using GH_3 cells (a rat pituitary gland epithelial tumor cell line) and TH, triiodothyronine (T_3). Furthermore, we also explored the co-relation of binding pattern and TR-mediated response [the production of prolactin (PRL)].

MATERIALS AND METHODS

Cell culture

GH_3 cells (a rat pituitary gland epithelial tumor cell line) [purchased from ATCC (Rockville, MD, U.S.A.)] were cultured with Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.) with 10% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, U.S.A.), glucose, L-glutamine, 110 mg/L sodium pyruvate, 10 mM N-2-hydroxy ethylpiperaxine-N'-2-ethane sulfonic acid (HEPES), 3.7 mg/mL $NaHCO_3$, 10 U/mL penicillin, and 10 μg/mL streptomycin (GIBCO BRL, Grand Island, NY, U.S.A.). All cells were maintained under a fully humidified atmosphere of 95% air- 5% CO_2 at 37°C. Cells were subcultured twice or three times a week.

Chemicals

Radio-labeled triiodothyronine (^{125}I-T_3, 2200 Ci/mmol) was obtained from Nuclear (Boston, MA, U.S.A.). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls (Aroclor 1254), bisphenol A (BPA), unlabeled T_3, triiodothyropropionic acid MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and monoiodotyrosine (MIT) were obtained from Sigma Chemical Co. (St. Louis, MD, U.S.A.).

Cytotoxicity assay

MTT assay was used to assess cell survival in a quantitative colorimetric assay (Cho *et al.*, 2000). MTT is reduced to blue-colored formazan by the mitochondrial enzyme succinate dehydrogenase, which is considered a reliable and sensitive parameter of mitochondrial function. Exponentially growing cells in the 24-well culture plates were treated with 10^{-11}~10^{-7} M T_3 with or without 10^{-12}~10^{-7} M TCDD in toluene, 10^{-11}~10^{-7} M aroclor 1254 in methanol, 10^{-11}~10^{-7} M BPA in ethanol. After 24 or 48 h, cells were incubated with 0.5 mL of MTT (1 mg/mL) for 30 min at 37°C. Then the MTT solution was removed, and cells were washed twice with PBS. The formazan formed in cells was extracted by adding 1 mL of isopropanol and incubating for 10 min at room temperature. The isopropanol was added directly to a plastic cuvette for spectrophotometric analysis at an absorbance wavelength of 570 nm.

Nuclear binding assay

Nuclear binding of ^{125}I-T_3 using GH_3 cells was examined as previously described (Samuels *et al.*, 1979), with some modification. Cells were plated into 24-well plate at a density of 2~5×10^5 cells/well and cultured for 1 day with serum-free medium (SFM). ^{125}I-T_3 was then added to the cells for the times indicated, and the plates were chilled to 0°C. Cells were rinsed twice with 1 mL PBS, and added with 100 μL of 0.05% trypsin, following 10 min incubation. Cells were suspended with 1 mL FBS, and spun down in 5 min at 800 × g. One milliliter of STM-Triton buffer containing 0.25 M sucrose, 20 mM Tris, 1.1 mM $MgCl_2$ and 0.5% Triton-X buffer (pH 7.85 at 25°C) was added to the plates for 20 min, and spun down in 10 min at 900 × g. Cells were rinsed twice with 2 mL STM-Triton buffer. Radioactivity of the nucleus was measured using gamma counter, and the amounts of nuclear bound T_3 were quantified. Scatchard analysis to calculate B_{max} and K_d values, binding sites/nucleus, and Lineweaver-Burk analysis to predict inhibitory mode of actions were conducted as reported previously (Amir *et al.*, 1978; Morgan and Grossman, 1984; Scatchard, 1949).

Enzyme immunoassay

GH_3 cells were plated at a density of 1×10^5 cells/well and cultured under serum free conditions for 24 h. Cells were then incubated with various concentrations of Aroclor 1254 and BPA in the presence of T_3. After 48 h incubation, secreted PRL was measured using Enzyme immunoassay, according to previous method (Maes *et al.*, 1997). Absorbance was measured at 450 nm.

Statistical analysis

The Student's *t*-test and an one-way ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data are expressed as means ± standard errors (SEM) and the results are taken from at least 3 independent experiments performed in triplicate. P values of 0.05 or less were considered to be statistically significant.

RESULTS

The effect of EDs and T_3 on the viability of GH_3 cells

To examine the effect of EDs on the viability of GH_3 cells, TCDD, Aroclor 1254 and BPA were treated to GH_3 cells for 48 h. As Fig. 2 shows, TCDD (10^{-12}~10^{-7} M), Aroclor 1254 (10^{-11}~10^{-6} M) and bisphenol A (10^{-9}~10^{-5} M) did not affect the viability of GH_3 cells. Higher concentration (100 μM) of bisphenol A, however, reduced GH_3 cell's viability up to 15%. Through the cell viability tests, therefore, the concentrations of EDs [10^{-12}~10^{-7} M (TCDD), 10^{-11}~10^{-6} M (Aroclor 1254), and 10^{-10}~5×10^{-4} M (BPA)] were used for further experiments. Furthermore, T_3 treatment did not drastically alter the viability of GH_3 cells up to 100 nM.

The effect of EDs on ^{125}I-T_3 nuclear binding in GH_3 cells

To study biochemical feature of EDs on T_3 binding to TR, ^{125}I-T_3 nuclear binding pattern in GH_3 cells was first examined under various time points to decide on the optimal incubation time. For this, cells were incubated with 1.6×10^{-10} M of ^{125}I-T_3 in the presence or the absence of 10^{-7} M of unlabeled T_3 for indicated times. As shown in Fig.

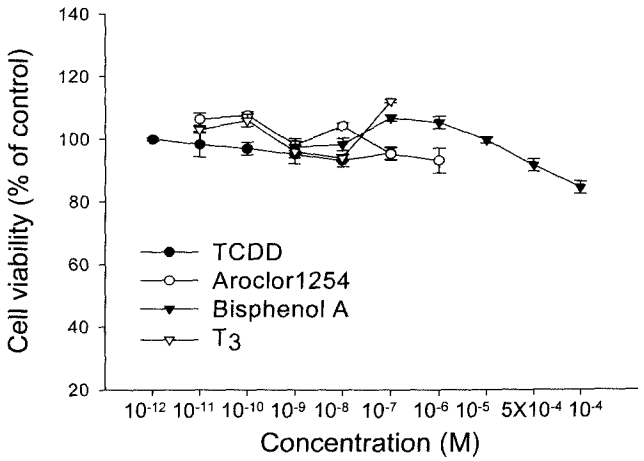

Fig. 2. Effect of EDs and T_3 on the cell viability of GH_3 cells. GH_3 cells (5×10^5 cells/well) were cultured with various concentrations of EDs and T3 for 48 h. Cell viability was determined by conventional MTT assay as described in Materials and Methods. The data represent mean± S.E.M of three independent experiments performed by triplicates.

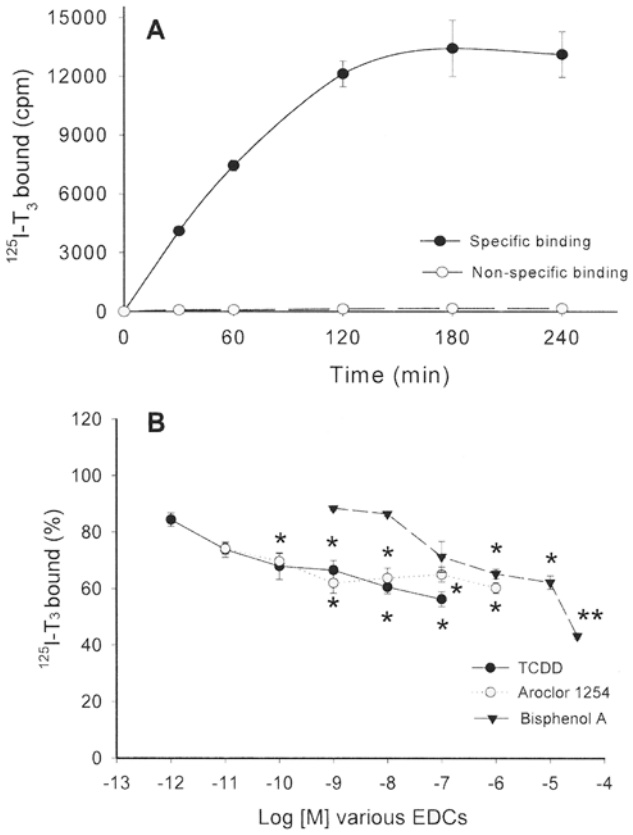

Fig. 3. ^{125}I-T_3 nuclear binding assay in GH_3 cells and its inhibition by EDs. (A) GH_3 cells (5×10^5 cells/well) were incubated at various times with 1.6×10^{-10} M ^{125}I-T_3 in the presence or the absence of 10^{-7} M unlabeled T_3. (B) GH_3 cells (5×10^5 cells/well) were incubated at 37°C with 1.6×10^{-10} M ^{125}I-T_3 and various concentrations of EDs (TCDD, aroclor 1254 or bisphenol A). The data represent mean ± S.E.M of three independent experiments performed by triplicates. *: $p<0.05$ and **: $p<0.01$ compared to ^{125}I-T_3 alone.

3A, the amount of ^{125}I-T_3 nuclear binding was increased up to 180 min and maintained maximum level up to 240 min in GH_3 cell, suggesting that the optimal and saturated incubation time is 180 min in GH_3 cells.

To analyze equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}) of ^{125}I-T_3 receptor binding, ^{125}I-T_3 saturation binding assay was conducted. The extent of ^{125}I-T_3 receptor binding was elevated in a ^{125}I-T_3 concentration-dependent manner up to 0.3 nM (data not shown). The values of K_d and B_{max} for ^{125}I-T_3 nuclear receptor binding were calculated by Scatchard analysis. As shown in Table I, GH_3 cells had the K_d value of $4.32 \pm 0.69\times10^{-10}$ M and the B_{max} value of 17.74 ± 0.75 fmol/5 × 10^5 cells. Meanwhile, PC12 cells displayed the K_d value of $1.34 \pm 0.28\times10^{-10}$ M and the B_{max} value of 0.82 ± 0.05 fmol/5×10^5 cells (data not shown).

Next, we elucidated the effect of EDs on TR binding of T_3. For this experiment, unlabeled T_3 and triiodothyropropionic acid (TITPA, T_3 analogue), and monoiodo-tyrosine (MIT, iodothyronine analogue) was used as positive or negative control drugs. After cells were treated with ^{125}I-T_3 (1.6×10^{-10} M) and various concentrations of EDs, TR binding assay was conducted. Receptor binding of T_3 was markedly reduced by treatment of unlabeled T_3 and TITPA, whereas the binding was rarely inhibited by MIT in GH_3 cells (data not shown). The treatment of TCDD, Aroclor 1254 and BPA significantly blocked T_3 binding in a dose-dependent manner up to about 44%, 40% and 57%, respectively (Fig. 3B).

Table I. K_d and B_{max} values and binding sites per cell nucleus in T_3-treated GH_3 cells

Parameter	Mean ± S.E
K_d (10^{-10} M)	4.32 ± 0.69
B_{max} (fmol/5×10^5 cells)	12.74 ± 0.75

Biochemical characterization of ED-mediated inhibition on T_3-TR binding

We next analyzed the effect of EDs on the T_3-TR binding affinity (K_d) and maximum binding (B_{max}). The alteration of K_d and B_{max} values and inhibitory mode of action were evaluated by Scatchard and Lineweaver-Burk plot analyses. As Fig. 4 and Table II show, TCDD (10^{-7} M) and aroclor 1254 (10^{-7} M) significantly increased the K_d value for the T_3 binding to TR, but these compounds did not influence on the B_{max} value, suggesting that these two EDs may be involved in suppressing T_3 binding by a competitive inhibition manner. In contrast, BPA (5×10^{-4} M) significantly decreased the B_{max} value but not the K_d value, suggesting this compound may inhibit T_3 binding in a non-competitive manner.

The effects of EDs on prolactin (PRL) production

There are numerous lines of evidence that EDs mimic the cellular action of TH via TR. Therefore, we next investigated whether an interruption of T_3 binding by EDs affects its biological function. To do this, effect of two EDs (aroclor 1254 and BPA) with different inhibitory mode of action on the TR-mediated cellular response such as the production of prolactin (PRL) was investigated. Since GH_3 cells treated with 10^{-7}~10^{-11} M of T_3 for 48 h produced PRL in a dose-dependent manner (data not shown), 1 nM of T_3 was chosen for further experiments with EDs.

Aroclor1254 exposure with T_3 significantly up-regulated the production of PRL at concentrations ranged from 0.01 to 100 nM (Fig. 5A). In contrast, interestingly, bisphenol A dose-dependently suppressed the production of PRL (Fig. 5B). Meanwhile, triiodothyroacetic acid (TAA) also increased the production of PRL up to 4 times more than normal culture condition.

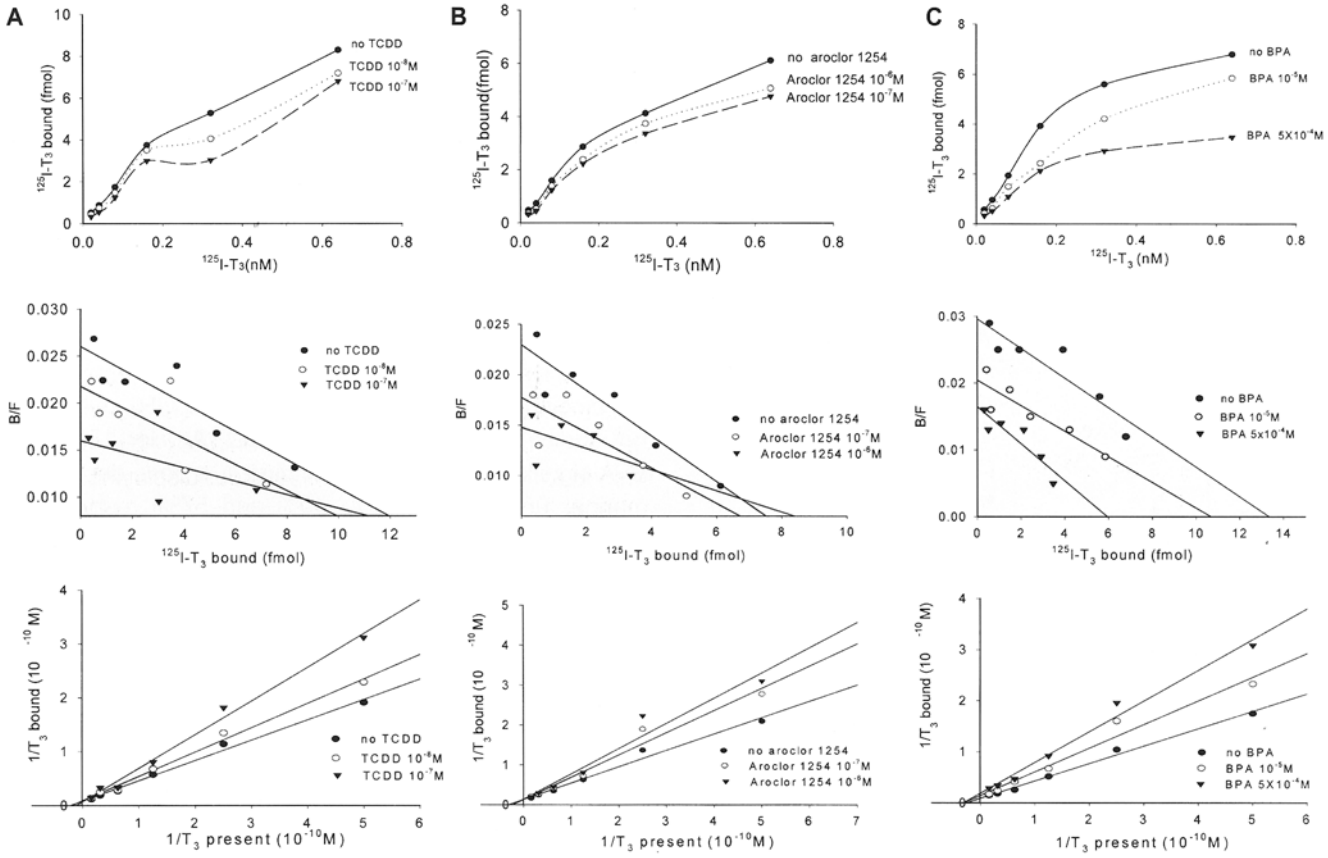

Fig. 4. Schachard plot and Lineweaver-Burk analysis of T_3 binding inhibition by TCDD (A), aroclor 1254 (B) or bisphenol A (C). (A, B and C : Upper panels) GH_3 cells were incubated in the presence of fixed concentrations of EDs and various concentrations of ^{125}I-T_3. The data were replotted by Scatchard (A, B and C : Middle panels) and Lineweaver-Burk analysis (A, B and C : Lower panels). The data represent mean of three independent experiments performed by triplicates.

Table II. Characteristics of the inhibition of binding of T_3 to GH_3 cell nucleus by various EDs (TCDD, aroclor 1254 and bisphenol A), from Scatchard plots and Lineweaver-Burk analysis

ED	Concentration (M)	B_{max} (fmol/5×10^5 cells)	K_d (10^{-10} M)
TCDD	0	17.76±1.47[1)]	6.94±0.99
	1.0×10^{-8}	18.43±4.70	8.78±2.61
	1.0×10^{-7}	18.81±1.93	11.53±1.20*
	Lineweaver-Burk analysis	Competitive inhibition	
Aroclor 1254	0	10.56±0.24	4.55±0.15
	1.0×10^{-7}	10.18±0.97	5.74±0.75
	1.0×10^{-6}	12.28±1.28	8.06±1.1*
	Lineweaver-Burk analysis	Competitive inhibition	
Bisphenol A	0	12.96±0.22*	4.38±0.19
	1.0×10^{-5}	11.76±1.10	5.89±0.76
	5.0×10^{-4}	6.61±0.54**	4.16±0.64
	Lineweaver-Burk analysis	Non-competitive inhibition	

1) Values are the mean ± S.D., n=3
* : $p<0.05$ and ** : $p<0.01$ compared to control alone

DISCUSSION

Thyroid hormones are involved in normal differentiation, growth, and metabolism in several tissues of all vertebrates (Hsieh and Juang, 2005; Incerpi, 2005). Their actions are mediated by TR that belongs to the superfamily of ligand-

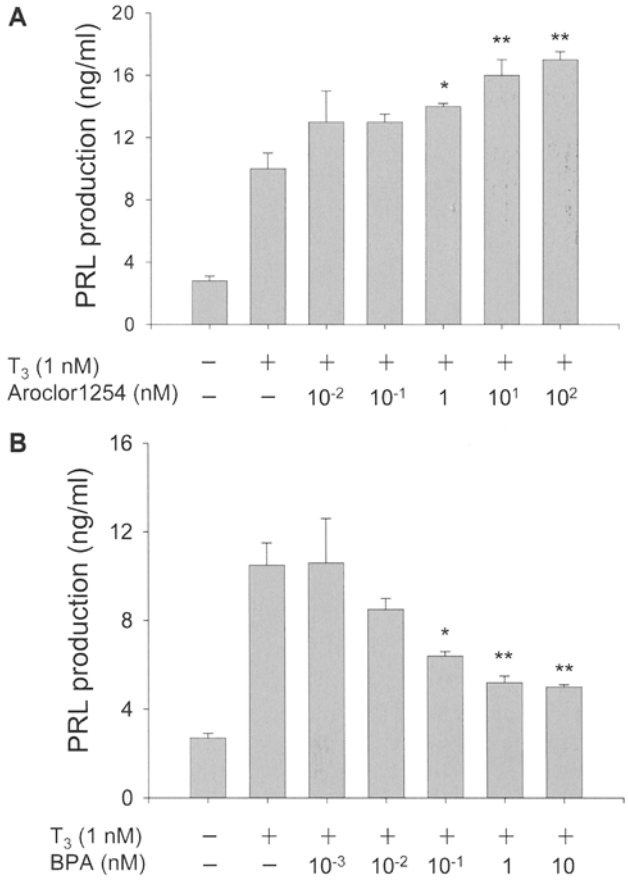

Fig. 5. Effect of aroclor 1254 (A) and BPA (B) on T_3-induced prolactin (PRL) production in GH_3 cells. Cells (1×10^5 cells/well) were incubated with various concentrations of aroclor 1254 (A) and BPA (B) in the presence of T_3. The determination of PRL secreted was carried out as described in Materials and Methods. The data represent mean± S.E.M of three independent experiments performed by triplicates. *: $p<0.05$ and **: $p<0.01$ compared to ^{125}I-T_3 alone.

dependent transcription factors (Hsieh and Juang, 2005; Incerpi, 2005). The TR directly regulates the transcription of thyroid hormone-responsive genes in response to changing levels of thyroid hormone via direct binding of the TR response element (TREs), in promoters of target genes (Gloss *et al.,* 2005). In particular, recently it has been known that TR interaction with TREs requires several proteins such as coregulatory proteins (co-repressor and co-activators), to provide specific local control of transcriptional responses (Matsushita *et al.,* 2000; Miyazaki *et al.,* 2004).

We, therefore, elucidated the effect of TCDD, Aroclor 1254 and BPA, which have similar structure with thyroid hormone (Moriyama *et al.,* 2002), on the regulation of TR function. First, nuclear binding of ^{125}I-T_3 was examined using TH-responsive GH_3 cells. As shown in Table I, GH_3 cells displayed large number of nuclear binding sites (21,000/nucleus) and maximum binding capacity (17.74 fmol/5×10^5 cells), compared to PC12 cells (nuclear binding sites : 1021/nucleus, and maximum binding capacity : 0.82 fmol/5×10^5 cells) (data not shown). The big difference of these parameters between two cell lines seems to be due to the receptor level. Thus, PC12 cells have been reported to dominantly express α_2 isoform of TR, which does not bind to thyroid hormone (Hohenwarter *et al.,* 1996), whereas GH_3 cells have TR β_2 isoform, known to bind to T_3 (Bernal and Nunez, 1995) (Table I). This result indicates that T_3's binding to TR in GH_3 cells is not due to simple non-specific binding to nucleus components.

As Fig. 1 shows, EDs and T_3/T_4 are structurally similar and EDs are also known to antagonize TR function (Naciff and Daston, 2004; Stoker *et al.,* 2000). In our experiments, we obtained that EDs dose-dependently blocked T3 binding to TR up to 40 to 50%, without displaying drug's cytotoxicity (Figs. 2 and 3). Even though Aroclor 1254 structurally more similar to bisphenol A than TCCD, however, these two EDs (Aroclor 1254 and bisphenol A) exhibited different biochemical properties in inhibiting T3 binding to TR. Thus, Aroclor 1254 blocked T_3 binding in a competitive inhibitory manner, judged by the dose-dependently increased K_d value, whereas bisphenol A showed a non-competitive fashion with the dose-dependently increased B_{max} value (Fig. 4 and Table II). Furthermore, TCDD (which has a less similar structure to T_3) and bisphenol A also inhibited competitively the binding of T_3 to TR, suggesting that subtle difference of ED structure may not be recognized by TR structure. Otherwise, it is also speculated that even the difference may be enough to interact with TR pocket (Asn331 in TR β isoforms) via hydrogen bonding, a critical binding force between TR and its ligand (Ye *et al.,* 2003). Considering that bisphenol A non-competitively blocked T_3 binding, there is a possibility that TR may have another ED binding site, distinguished from T_3 binding pocket. However, since different binding affinity can also cause different inhibitory patterns to the receptor (Katz and Lazar, 1993), more careful study should be followed to verify several possibilities regarding ED's binding to TR.

PRL expression is known to be stimulated by various hormones such as estrogen (Sharp *et al.,* 2006), thyrotropin releasing hormone (Murdoch *et al.,* 1983), epidermal growth factor (EGF) (Hnasko and Ben-Jonathan, 2003) and T_3 (Williams *et al.,* 1991). Because GH_3 cells are one of representative cell lines producing PRL by T_3 (Carmeliet *et al.,* 1989), we finally examined the effect of two EDs with distinct inhibitory feature on the T_3-mediated production of PRL, to obtain a correlation between biochemical property and biological action. As Fig. 5 shows, Aroclor 1254 and BPA distinctly modulated PRL production in GH_3 cells stimulated by T_3 for 48 h. Thus, Aroclor 1254 strongly enhanced PRL production in a dose-dependent manner, whereas BPA dose-dependently diminished the production

of PRL, suggesting that ED binding pattern to TR and binding affinity between ED and TR may be critical to decide the regulatory role of EDs in TR-mediated responses. In particular, considering that 10 nM of BPA blocked almost 40% of T_3 binding, BPA binding to TR may trigger allosteric conformational change to down-regulate T_3 binding, resulting in lowered TR response, as shown in the case of the regulation by coactivator of TR (Liu *et al.*, 1998). Moreover, our results regarding Aroclor 1254-mediated up-regulation of PRL production indicate that Aroclor 1254 itself may possess T_3-like property. Indeed, this compound has been demonstrated to induce the production of various hormones from thyroid and adrenaline (Hallgren *et al.*, 2001).

In conclusion, we found that TCDD, Aroclor 1254 and BPA significantly blocked T_3 binding to TR in a dose-dependent manner. Biochemical characterization by Scatchard and Lineweaver-Burk plot analyses indicated that TCDD and Aroclor 1254 bound to TH receptors in a competitive inhibitory manner, whereas BPA bound to TH receptors in a non-competitive pattern. Interestingly, Aroclor 1254 exposure for 48 h enhanced T_3-mediated PRL production, but BPA down-regulated. These results suggest that the EDs (TCDD, Aroclor 1254 and BPA) could differentially bind to TR and distinctly regulate the action of TR function, even though the EDs are structurally similar.

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