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Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A☆

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Abstract

The thyroid hormonal-disrupting activity of the flame retardants tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) was examined and compared with that of bisphenol A, a typical estrogenic xenobiotic. TBBPA and TCBPA, halogenated derivatives of bisphenol A, markedly inhibited the binding of triiodothyronine (T_3; 1×10^{-10} M) to thyroid hormone receptor in the concentration range of 1×10^{-6} to 1×10^{-4} M, but bisphenol A did not. The thyroid hormonal activity of TBBPA and TCBPA was also examined using rat pituitary cell line GH3 cells, which grow and release growth hormone (GH) depending on thyroid hormone. TBBPA and TCBPA enhanced the proliferation of GH3 cells and stimulated their production of GH in the concentration range of 1×10^{-6} to 1×10^{-4} M, while bisphenol A was inactive. TBBPA, TCBPA, and bisphenol A did not show antagonistic action, i.e., these compounds did not inhibit the hormonal activity of T_3 to induce growth and GH production of GH3 cells. TBBPA and TCBPA, as well as bisphenol A, enhanced the proliferation of MtT/E-2 cells, whose growth is estrogen-dependent. These results suggest that TBBPA and TCBPA act as thyroid hormone agonists, as well as estrogens. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Thyroid hormonal activity; Estrogenic activity; Rat pituitary tumor cell line GH3; Rat pituitary tumor cell line MtT/E-2; Tetrabromobisphenol A; Tetrachlorobisphenol A; Bisphenol A

Tetrabromobisphenol A (2,2-bis-(3,5-dibromo-4-hydroxyphenyl)-propane; TBBPA) and tetrachlorobisphenol A (2,2-bis-(3,5-dichloro-4-hydroxyphenyl)-propane; TCBPA) are halogenated derivatives of bisphenol A (BPA), a typical xenoestrogen. These halogenated compounds, especially TBBPA, are widely used throughout the world as flame retardants for building materials, paints, synthetic textiles, and plastic products, including epoxy resin electronic circuit boards and other electronic equipment. TBBPA and TCBPA were developed as a new, safe class of flame retardants because they are not readily accumulated in the environment and are not highly toxic [1]. TBBPA currently accounts for about one-third of the total usage of flame retardants. Despite the fact that TBBPA is usually chemically bound to a substrate, its high usage and limited water solubility may be expected to lead to persistence in the environment and possibly accumulation in biological systems. TBBPA and its dimethylated derivative have been found in river sediment in Osaka, Japan at concentrations of 0.5–140 μg/kg dry weight [2]. These compounds were also detected in sites downstream from a plastics production facility at 270 ng/g dry weight as TBBPA and at 1500 ng/g dry weight as the dimethyl derivative, and in sewage sludge samples in Sweden [3]. The halogenated compound was also found in air samples at an electronics recycling plant [4]. Recently, Thomsen et al. [5] detected TBBPA and TCBPA in human plasma lipids at the level of 4–200 pg/g plasma.

☆ *Abbreviations*: TBBPA, tetrabromobisphenol A; TCBPA, tetrachlorobisphenol A; BPA, bisphenol A; T_3; L-3,5,3′-triiodothyronine; GH, growth hormone; HS, horse serum; FBS, fetal bovine serum.

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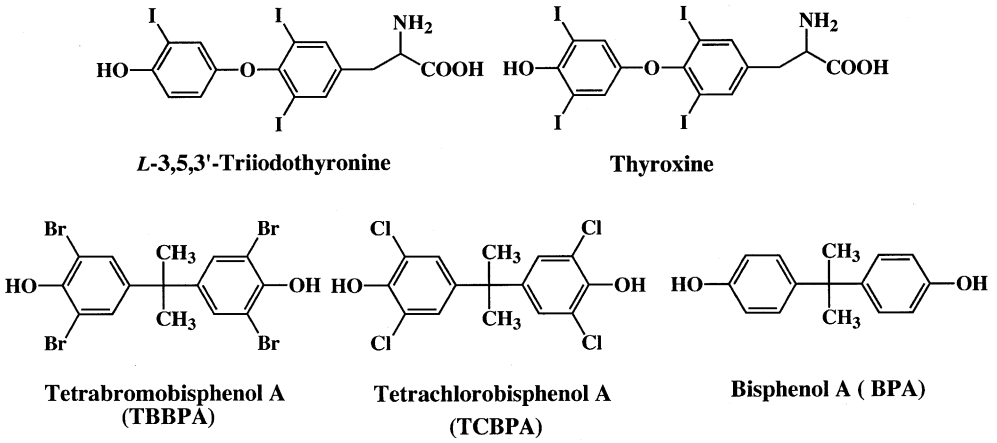

Fig. 1. Structures of tetrabromobisphenol A, tetrachlorobisphenol A, bisphenol A, and thyroid hormones.

Some man-made chemicals which are widely distributed in the environment are able to mimic the biological activity of hormones, and are known as endocrine-disrupting chemicals. These chemicals include chlorinated insecticides, such as kepone, *o*,*p*′-DDT, dieldrin and methoxychlor, and nonchlorinated compounds used in the plastics and detergent industries, such as alkylphenols and BPA, as estrogenic compounds [6]. *p*,*p*′-DDE, a metabolite of *p*,*p*′-DDT, and vinclozolin, an antifungal agent, are known to have anti-androgenic activity [7–9]. Some hydroxy-PCBs such as 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl are reported to show anti-thyroid hormonal activity in addition to estrogenic activity [10–13]. Interactions of estrogenic and anti-androgenic compounds with the respective hormone receptors have been demonstrated to account for the endocrine-disrupting actions. However, the exact mechanisms of interference with thyroid hormonal action are not fully understood.

It is well known that BPA, an industrial raw material for polycarbonate and epoxy resins, shows estrogenic activity [14,15]. However, the hormonal activity of the halogenated derivatives, TBBPA and TCBPA, has not been examined. In this report, the thyroid hormonal and anti-thyroid hormonal activities of TBBPA, TCBPA, and BPA (Fig. 1) were examined by means of binding assay with thyroid hormone receptor, as well as thyroid hormone-dependent growth assay and production of growth hormone (GH) in pituitary cell line GH3 cells. We demonstrate that TBBPA and TCBPA exhibit significant thyroid hormonal activities, as well as estrogenic activity. In contrast, BPA showed no thyroid hormonal activity, in spite of its estrogenic activity.

Materials and methods

Chemicals. TBBPA (>98%), TCBPA (>98%) BPA (>99%) were obtained from Tokyo Chemical Industry (Tokyo, Japan), and L-3,5,3′-triiodothyronine (T_3; 98%), 17-β-estradiol (>98%), and tamoxifen (>99%) from Sigma Chemical (St. Louis, MO). ^{125}I-T_3 (3,5,3′-^{125}I, radiochemical purity >95%, 28.8 TBq/mmol) and ^{3}H-17-β-estradiol (2,4,6,7-^{3}H, radiochemical purity >97%, 3.4 TBq/mmol) were purchased from NEN Life Science Products, Boston, MA.

Cell culture. The thyroid hormone-responsive pituitary cell line GH3 was maintained in DME/F12 mixed medium (Sigma Chemical) containing penicillin and streptomycin with 15% horse serum (HS, Life Technologies, Rockville, MD) and 2.5% fetal bovine serum (FBS). For the estrogen-responsive pituitary cell line MtT/E-2, 8% HS and 2% FBS were used. Before cell growth assay and GH production assay, cells were maintained for 2–3 days in phenol-red free DEM/F12 (Sigma Chemical) containing the same antibiotics along with dextran–charcoal treated HS and FBS.

Competitive binding assay to thyroid hormone receptor. MtT/E-2 cells were homogenized in 0.32 M sucrose solution containing 3 mM $MgCl_2$ and 1 mM dithiothreitol and centrifuged at 700*g* for 10 min. The pellets were resuspended in 2.4 M sucrose with $MgCl_2$ and centrifuged at 53,000*g* for 45 min. The resulting nuclear pellets were resuspended in TMDS buffer (2 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, and 0.32 M sucrose, pH 7.4). Various concentrations of test chemicals and 3 nM ^{125}I-T_3 were incubated in 0.2 ml of the nuclear suspension at 37 °C for 40 min. After incubation, 0.25 ml of 2% Triton X-100 was added to terminate the reaction, and mixture was centrifuged at 1000*g* for 10 min. The pellets were washed two times with 1 ml of TMDS buffer and the supernatant was removed. Radioactivity of the pellets was counted with a gamma counter (Wallac Wizard 1480, Perkin–Elmer Life Sciences, Boston, MA).

Competitive binding assay to estrogen receptor. MtT/E-2 cells were homogenized with TEDMG buffer (1 mM disodium EDTA, 1 mM dithiothreitol, 10 mM sodium molybdate, 10% (v/v) glycerol, and 10 mM Tris–HCl, pH 7.4). The cytosolic fraction was obtained from the homogenate by centrifugation at 105,000*g* for 60 min. For the assay, 0.1 ml of the cytosolic fraction was added to the same volume of TEDMG buffer containing 0.5 nM ^{3}H-17-β-estradiol and various concentrations of test compounds. After incubation at 30 °C for 40 min, 0.05 ml of 1.5% charcoal, and 0.15% dextran T70 mixture was added. After incubation for 10 min at 4 °C with occasional vortexing, the suspension was centrifuged at 800*g* for 10 min. A 0.1 ml aliquot of the supernatant was transferred to another tube, and 1 ml of scintillator was added. The radioactivity in each tube was counted with a Wallac Micro-Beta Scintillation Counter (Perkin–Elmer Life Sciences).

GH production assay in GH3 cells. The cells were seeded in 24-well plates at 1×10^4 cells/well and chemicals were added the next day. Two days later, growth hormone in the culture medium was measured by

radioimmunoassay with NIADDK reagents following the recommended protocol. The details were previously reported [16].

Thyroid hormone-dependent growth assay of GH3 cells and estrogen-dependent growth assay of MtT/E-2 cells. GH3 cells and MtT/E-2 cells were seeded in 24-well plates at 1×10^4 and 3×10^3 cells/well, respectively, and chemicals dissolved in 10 μl of ethanol were added on the following day. One week later, cell growth was measured with a modified MTT assay kit, which employs a newly developed tetrazolium salt, WST-1 (Dojindo Chemicals, Kumamoto, Japan). The details were previously reported [17].

Results

Competitive binding assay for thyroid hormone-like compounds

The inhibitory effects of TBBPA, TCBPA, and BPA on binding of T_3 to thyroid hormone receptor were examined. T_3 competitively inhibited the binding of ^{125}I-T_3 (1×10^{-10} M) to thyroid hormone receptor in the range of 1×10^{-9} to 1×10^{-6} M. TBBPA and TCBPA also markedly inhibited the binding of ^{125}I-T_3 to the receptor in the range of 1×10^{-6} to 1×10^{-4} M. However, little effect was observed with BPA. When the nuclear fraction was boiled, the binding of these compounds was not observed (see Fig. 2).

Thyroid hormonal activity of TBBPA and TCBPA evaluated by growth assay of GH3 Cells

The thyroid hormonal activity of TBBPA and TCBPA was also examined by assay of thyroid hormone-dependent growth of GH3 cells. The growth-inducing effect of T_3 on the cells was observed over the range of 1×10^{-11} to 1×10^{-9} M. When TBBPA and TCBPA were added to the cells, growth was also stimulated at 1×10^{-6} to 1×10^{-4} M in the case of TBBPA, and at 1×10^{-4} M in the case of TCBPA (Fig. 3A).

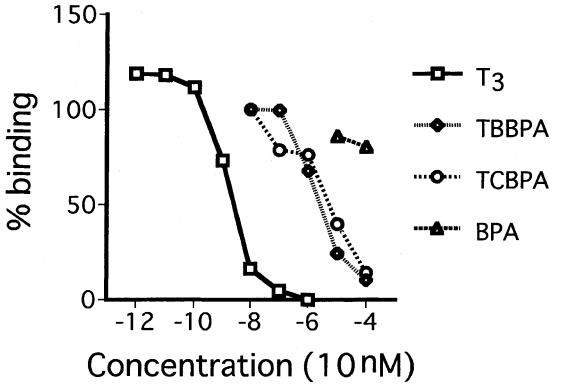

Fig. 2. Binding assay of test compounds to thyroid hormone receptor. Each value represents the mean of duplicate determinations. Activity was expressed relative to the control with no added test compound. T_3; L-3,5,3′-triiodothyronine, TBBPA; tetrabromobisphenol A, TCBPA; tetrachlorobisphenol A, BPA; bisphenol A.

These results indicate that TBBPA and TCBPA show thyroid hormone-like activity.

Thyroid hormonal activity of TBBPA and TCBPA evaluated by assay of GH production by GH3 cells

The thyroid hormonal activities of TBBPA and TCBPA were further examined by measuring the ability of these compounds to induce the production of GH by GH3 cells. GH release activity was observed with T_3 in the range of 1×10^{-11} to 1×10^{-9} M. When GH release from GH3 cells was measured after the addition of TBBPA or TCBPA, an increase was also observed in the range of 1×10^{-6} to 1×10^{-4} M (Fig. 3B). These results are consistent with those in the case of cell growth assay, described above.

Anti-thyroid hormonal activity of TBBPA, TCBPA, and BPA in GH3 cells

The inhibitory effects of TBBPA, TCBPA, and BPA on the hormonal activity of T_3 on GH3 cells were examined. These compounds at concentrations of 1×10^{-5} and 1×10^{-4} M did not inhibit the induction of GH3 cell growth by 1×10^{-10} and 1×10^{-9} M T_3 (Figs. 4A and B). These compounds at 1×10^{-5} and 1×10^{-4} M also did not show antagonistic action towards GH production induced by the thyroid hormone (Figs. 4C and D). These results suggest that TBBPA and TCBPA act as thyroid hormone agonists, but not antagonists.

Estrogenic and anti-estrogenic activities of TBBPA, TCBPA, and BPA

The estrogenic activities of TBBPA, TCBPA, and BPA were examined by using binding assay to estrogen receptor and assay of estrogen-dependent growth of MtT/E-2 cells. TBBPA, TCBPA, and BPA competitively inhibited the binding of ^{3}H-β-estradiol (1×10^{-10} M) to estrogen receptor in MtT/E-2 cells in the range of 1×10^{-5} to 1×10^{-4} M (Fig. 5A). Furthermore, estrogenic activity was examined using MtT/E-2 cells, whose growth is stimulated by estradiol [17,18]. When these compounds were added to the cells, growth was stimulated in the range of 1×10^{-6} to 1×10^{-4} M. BPA showed the highest activity, followed by TCBPA and TBBPA (Fig. 5B). The estrogenic activities of these compounds were markedly inhibited by the addition of tamoxifen (1×10^{-7} M). In contrast, no inhibitory effect of TBBPA and TCBPA (1×10^{-6} to 1×10^{-4} M) on the estrogenic activity of β-estradiol at the concentration of 1×10^{-11} M was observed in growth assay using MtT/E-2 cells (data not shown). These experiments indicate that the order of estrogenic activities of these compounds is opposite to that of the thyroid hormonal activities, but TBBPA and TCBPA are both estrogenic, as is BPA.

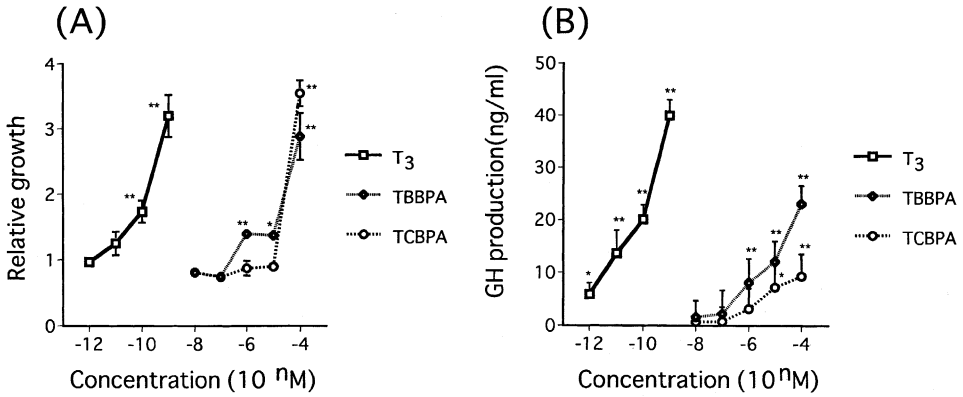

Fig. 3. Thyroid hormonal activity of tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) in GH3 cells. Thyroid hormone-dependent growth of GH3 cells (A). Growth hormone (GH) release from GH3 cells (B). Each bar represents the means ± SD of four experiments. Activity was expressed relative to the control untreated GH3 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

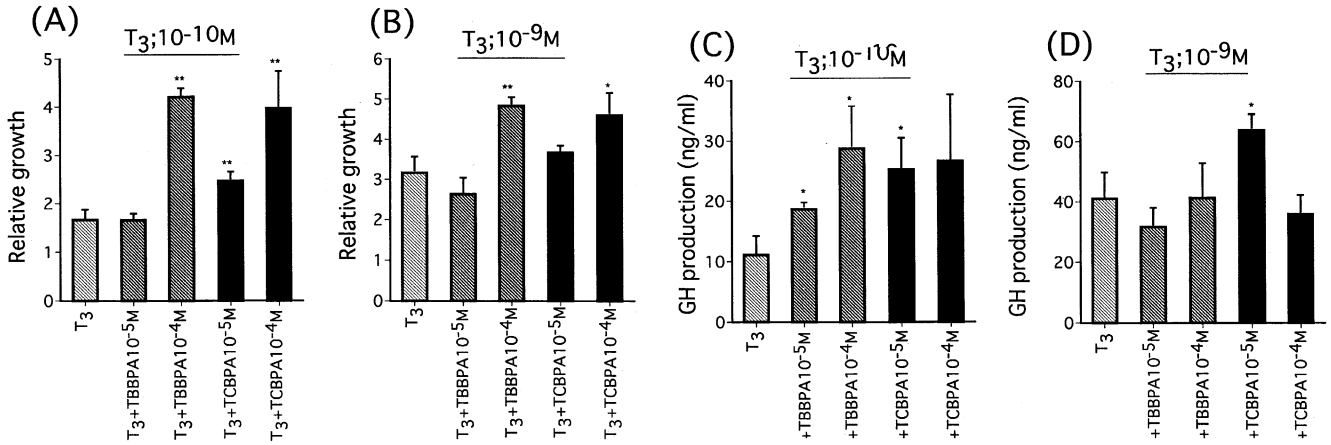

Fig. 4. Anti-thyroid hormonal activity of tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) in GH3 cells. Anti-thyroid hormonal activity of these compounds against the activity of L-3,5,3′-triiodothyronine (T_3) at the concentrations of 1×10^{-10} M (A) and 1×10^{-9} M (B) was evaluated by growth assay of GH-3 cells. Anti-thyroid hormonal activity of these compounds against the activity of T_3 at the concentrations of 1×10^{-10} M (C) and 1×10^{-9} M (D) was also evaluated in terms of growth hormone release from GH3 cells. Each bar represents the means ± SD of four experiments. Activity was expressed relative to the control. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

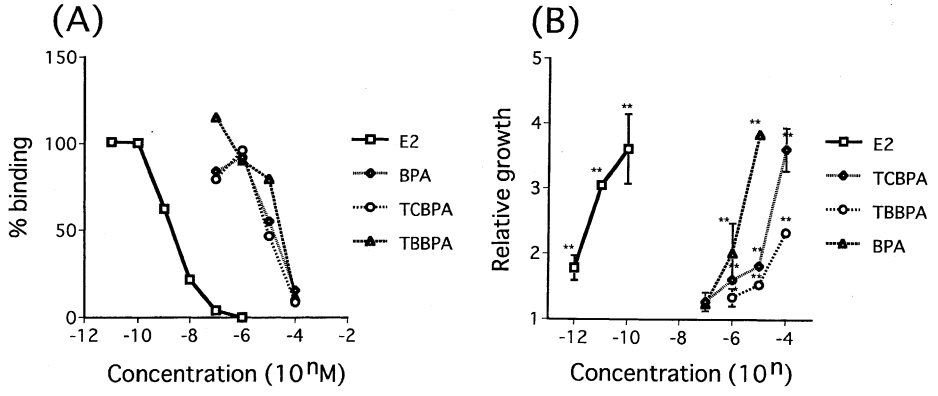

Fig. 5. Estrogenic activity of tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA), and bisphenol A (BPA). Binding assay to estrogen receptor (A). Estrogen-dependent growth assay of MtT/E-2 cells (B). Each value represents the mean of duplicate determinations in (A) and the means ± SD of four experiments in (B). Estrogenic activity was expressed relative to the control untreated MtT/E-2 cells in (B). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. E2; 17-β-estradiol.

Discussion

TBBPA is currently the most widely used flame retardant, and its residues have been detected in wildlife and humans [2–4]. TBBPA shows acute toxicity toward mysids (*Mysidopsis bahia*), but its toxicity is much lower than that of malathion or tributyltin [19]. No acute toxicity of TBBPA to aquatic organisms such as zebrafish and waterflea has been detected [20,21]. Szymanska et al. [22] reported that oral administration of TBBPA tended to decrease the activity of 5-aminolevulinate synthase, though α-glutamyl transferase activity and the level of cytochrome P450 did not show any significant change. They suggested that TBBPA may disturb heme metabolism in rats. Here, we present the first evidence that TBBPA and TCBPA have thyroid hormonal activity, though BPA does not.

There are at least three different levels at which environmental contaminants interact with the thyroid hormone system. These are toxicity at the thyroid gland, disturbance of thyroid hormone metabolism, and interaction with thyroid hormone transport proteins. A number of chemicals have been reported to bind to transthyretin, one of the thyroid hormone-binding transport proteins in plasma of vertebrate species. Some halogenated compounds bind to human transthyretin and thyroid-binding globulin in vitro, competing with thyroid hormone binding [10,13,23]. It was demonstrated that bromine substituents play a crucial role in the binding potency with transthyretin. Cheek et al. [13] reported that some hydroxylated PCBs inhibit the binding of T_3 to recombinant human TRβ expressed in Sf9 cells. TCBPA is also reported to bind with transthyretin, though with lesser potency than TBBPA [23]. In the current study, it was shown that TBBPA and TCBPA interact with thyroid hormonal receptor. These results suggest that TBBPA and TCBPA have the ability to disrupt thyroid hormonal activity via two different mechanisms in vivo.

In this study, the thyroid hormonal potency of TBBPA and TCBPA was examined, in view of their structural resemblance to the thyroid hormones. We found that TBBPA and TCBPA show thyroid hormonal activity, though BPA does not. It is important to understand the structural requirements for thyroid hormonal activity in xenobiotics. A 4-hydroxyl group and two adjacent 3,5-halogen substituents in the phenyl group are essential factors. Relatively large substituents at the 3,5-position of the phenyl ring bearing the 4-hydroxy group also seem to be necessary for thyroid hormonal activity. Various polybrominated biphenyls, polybrominated diphenyl ethers, or their metabolites may compete with thyroid hormones for binding to the thyroid hormone receptor.

We also tested whether BPA and the halogenated derivatives exhibit estrogenic activity. Christiansen et al. [24] reported that the vitellogenin level of male rainbow trout after intraperitoneal injection of TBBPA did not increase. In contrast, we showed that TBBPA and TCBPA are estrogenic by means of estrogen receptor binding and growth stimulation in a rat pituitary tumor cell line MtT/E-2 cell line, whose growth is stimulated by estrogens [17,18]. The results suggest that these compounds exhibit endocrine disrupting action via two hormonal activities in animals. Hydroxy-PCBs were also reported to show both estrogenic and anti-thyroid hormonal activities [11–13]. In the present study using GH3 cells, we observed agonistic activities of TBBPA and TCBPA toward thyroid hormone, but we could not detect anti-thyroid hormonal action of TBBPA and TCBPA. Rat pituitary cell line GH3, isolated from a rat pituitary tumor, has been widely used as a standard pituitary cell model. The cell proliferation as well as growth hormone secretion have been shown to depend markedly on thyroid hormones, but little on estrogen [25,26]. We are currently examining the thyroid and anti-thyroid hormonal activities of TBBPA and TCBPA in other assay systems. BPA exhibited the highest estrogenic activity among the tested compounds, followed by TCBPA and TBBPA. In contrast, TBBPA exhibited the highest thyroid hormonal activity. TCBPA showed both activities at moderate levels. Thyroid hormonal and estrogenic activities of TBBPA and TCBPA observed in vitro may reflect endocrinal toxicity in vivo. The influence of TBBPA on thyroid hormone levels in vivo is currently being examined in rats treated with propylthiouracil, an anti-thyroid hormonal agent, in our laboratory.

Acknowledgments

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