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In Vitro and *In Vivo* Analysis of the Thyroid System–Disrupting Activities of Brominated Phenolic and Phenol Compounds in *Xenopus laevis*

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We investigated the effects of the brominated phenolic and phenol compounds, some of which are brominated flame retardants, on the binding of ^{125}I-3,3′,5-L-triiodothyronine (^{125}I-T_3) to purified *Xenopus laevis* transthyretin (xTTR) and to the ligand-binding domain of *X. laevis* thyroid hormone receptor β (xTR LBD), on the induction of a T_3-responsive reporter gene in a recombinant *X. laevis* cell line (XL58-TRE-Luc) and on T_3-induced or spontaneous metamorphosis in *X. laevis* tadpoles. Of the brominated phenolic and phenol compounds tested, 3,3′,5-tribromobisphenol A and 3,3′-dibromobisphenol A were the most potent competitors of ^{125}I-T_3 binding to xTTR and the xTR LBD, respectively. Structures with a bromine in either *ortho* positions with respect to the hydroxy group competed more efficiently with T_3 binding to xTTR and the xTR LBD. 3,3′,5-Tribromobisphenol A and 3,3′,5,5′-tetrabromobisphenol A, at 0.1–1.0μM, exerted both T_3 agonist and antagonist activities in the T_3-responsive reporter gene assay. Sera obtained from fetal bovine and bullfrog tadpoles weakened the T_3 agonist and antagonist activities of 3,3′,5-tribromobisphenol A, but not the T_3 antagonist activity of *o-t*-butylphenol, for which xTTR has no significant affinity. The T_3 agonist and antagonist activities of 0.5μM 3,3′,5-tribromobisphenol A were confirmed in the *in vivo*, short-term gene expression assay in premetamorphic *X. laevis* tadpoles using endogenous, T_3-responsive genes as molecular markers. Our results suggest that 3,3′,5-tribromobisphenol A affects T_3 binding to xTTR and xTR and that it interferes with the intracellular T_3 signaling pathway.

Key Words: **thyroid hormone; transthyretin; thyroid hormone receptor; brominated phenolic compounds; thyroid system–disrupting chemicals; *Xenopus laevis*.**

Halogenated organic compounds are released widely in the environment (Monirith *et al.*, 2003) and because of their persistent nature are of great concern to the health of humans and wildlife. These compounds are produced during the commercial production of industrial chemicals as by-products or during combustion processes (Vallack *et al.*, 1998). As most halogenated organic compounds are highly hydrophobic, they accumulate in the sediment of water environments near industrial zones and in the tissues (JEA, 1999), which have high lipid content, of organisms. Of the halogenated organic compounds, the brominated compounds are produced as flame retardants (Legler and Brouwer, 2003). Of the flame retardants, tetrabromobisphenol A has one of the highest production volumes (Birnbaum and Staskal, 2004). Bromophenols are used as a reactive flame-retardant intermediate or as a wood preservative (WHO, 2005). Bromophenols are also generated from the chlorination of wastewater containing bromide ions (Watanabe *et al.*, 1984) and from the biodegradation of other brominated compounds, such as brominated benzenes and some brominated diphenyl ethers (WHO, 2005).

To date, the effects of endocrine-disrupting chemicals on the sex steroid system have been mostly studied because of the controversy regarding the possible link between endocrine-disrupting chemicals and infertility, breast cancer, and low sperm counts in mammals including human beings. Therefore, the effects of endocrine-disrupting chemicals on the thyroid system have received comparatively little attention. However, a number of halogenated organic compounds are known to interfere with the thyroid system (Brucker-Davis, 1998). Recent studies have indicated that brominated diphenyl ethers, bisphenols, and phenols with a greater degree of bromination were potent competitors of L-thyroxine (T_4) binding to human transthyretin (TTR), one of the thyroid hormone (TH)–binding proteins in plasma (Meerts *et al.*, 2000). These results suggested that these brominated compounds can interfere with TH homeostasis *in vivo* at the extracellular level of THs. Subsequent studies (Kitamura *et al.*, 2002, 2005) have demonstrated that tetrabromobisphenol A, tetramethylbisphenol A, and 3,3′-dimethylbisphenol A, ranging in concentration from 10^{-7} to 10^{-4}M, acted as competitors of 3,3′,5-L-triiodothyronine (T_3) binding to rat thyroid hormone receptors (TRs) and that tetrabromobisphenol A exerted both T_3 agonist and antagonist activities on the T_3-dependent cell growth and growth

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hormone release assays in rat GH3 cells. Although tetrabromobisphenol A (10^{-6}–10^{-5}M) also exhibited T_3 antagonist activity in a T_3-responsive reporter gene assay using Chinese hamster ovarian cells, tetrabromobisphenol A (10^{-8}–10^{-4}M) failed to exert T_3 agonist activity in this cultured cell system (Kitamura *et al.*, 2005). The contradictory observations of tetrabromobisphenol A and its ability to elicit T_3 agonist activity in the two cultured cell systems are yet to be resolved.

Possible sites in the thyroid system that the organohalogens target include the thyroid gland (Collins *et al.*, 1977), TTR in plasma (Brouwer *et al.*, 1998), TH metabolism (Byrne *et al.*, 1987), and TR-regulated gene expression (Miyazaki *et al.*, 2004). There are a few environmental chemicals, at concentrations less than 10^{-6}M, that compete with T_3 binding to the TRs (Cheek *et al.*, 1999; Ishihara *et al.*, 2003). This suggests that sites in the thyroid system other than T_3 binding to the TRs are mainly targeted by the environmental chemicals, although this assumption is based on the results of *in vitro* studies (Cheek *et al.*, 1999; Gauger *et al.*, 2004; Ishihara *et al.*, 2003; Yamauchi *et al.*, 2003).

In the present study, we investigated the effects of the brominated phenolic and phenol compounds on the *Xenopus laevis* thyroid system *in vitro* and *in vivo*. *In vitro* assays include the competitive interactions of the chemicals with ^{125}I-T_3 binding to *Xenopus laevis* transthyretin (xTTR) and the ligand-binding domain of *X. laevis* thyroid hormone receptor β (xTR LBD) and the T_3-responsive reporter gene assay using an amphibian cell line permanently transduced with a lentiviral vector containing a T_3-responsive luciferase gene (Sugiyama *et al.*, 2005). The *in vivo* assay was a short-term gene expression assay in a tadpole (Kudo and Yamauchi, 2005). Of the potent chemicals detected in the *in vitro* assays, 3,3′,5-tribromobisphenol A exerted T_3 antagonist and agonist activities in the *in vivo* assay.

MATERIALS AND METHODS

Reagents. ^{125}I-T_3 (122 MBq/μg; carrier free) was purchased from NEN Life Science Products (Boston, MA). Unlabeled T_3 (> 98% purity) and T_4 (> 98% purity) were obtained from Sigma (St Louis, MO). 2,6-Dibromophenol (97% purity), 2,4,6-tribromophenol (97% purity), pentabromophenol (98% purity), and bisphenol A (> 98% purity) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3,3′,5,5′-Tetrabromobisphenol A (> 98% purity) was from Tokyo Kasei (Tokyo, Japan). 3-Bromobisphenol A (98% purity), 3,3′-dibromobisphenol A (98% purity), 3,5-dibromobisphenol A (98% purity), and 3,3′,5-tribromobisphenol A (98% purity) were synthesized as described below. 3,3′,5-trichlorobisphenol A (> 98% purity) was synthesized as described previously (Fukazawa *et al.*, 2001). AG 1-X8 resin was from BioRad (Hercules, CA). All other chemicals used in this study were either chromatography grade or the highest grade available and were purchased from either Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

All chemicals tested as thyroid system–disrupting chemicals were dissolved in dimethylsulfoxide to a concentration of 10mM. These chemicals were diluted with an appropriate buffer to give less than 0.4% (vol/vol) solvent. A control assay without the test chemicals was performed in the presence of the solvent at a concentration less than 0.4% (vol/vol). The solvent (0.4%) did not affect the competitive ^{125}I-T_3–binding assays (without solvent vs. with solvent: 100.0 ± 1.5% vs. 95.4 ± 1.8%, $n = 3$, for xTTR binding and 100.0 ± 1.2% vs. 96.8 ± 0.6%, $n = 3$, for xTR binding) or the T_3-responsive reporter gene assay (100.0 ± 1.7% vs. 102.8 ± 2.0%, $n = 3$).

Synthesis of brominated derivatives of bisphenol A. A solution of bisphenol A (1.2 g) in acetone (20 ml) was added to an aqueous solution of potassium bromide (2.4 g) in water (150 ml). To this mixture, an aqueous solution of sodium hypochlorite (1%, 50 ml) was added drop by drop with stirring. The mixture was kept at about pH 3 by the addition of 1M hydrochloric acid and was stirred for 3 h. The reaction mixture was extracted with ethyl acetate. The organic solution was washed with brine and dried over anhydrous magnesium sulfate. After removal of the solvent under reduced pressure, the residue was subjected to silica gel column chromatography (20% ethyl acetate in hexane) to give 3-bromobisphenol A (270 mg), 3,3′-dibromobisphenol A (310 mg), 3,5-dibromobisphenol A (154 mg), and 3,3′,5-tribromobisphenol A (140 mg). These products were identified by nuclear magnetic resonance (NMR). NMR spectra were obtained on a JEOL JNM-GSX500 Fourier-transform spectrometer with tetramethylsilane as an internal standard.

Preparation of recombinant xTTR and xTR LBD. Recombinant xTTR and the xTR LBD–fused glutathione-S-transferase (GST) were expressed in *Escherichia coli* BL21 and purified from the bacterial extracts by affinity column chromatography. Recombinant xTTR was purified using human retinol–binding protein coupled to Sepharose 4B (Larsson *et al.*, 1985), and the GST–xTR LBD fusion protein was purified using glutathione coupled to Sepharose 4B (GE Healthcare Bio-Sciences, Piscataway, NJ) as described previously (Yamauchi *et al.*, 2002). SDS-PAGE showed that the purified xTTR and GST-xTR LBD fractions contained a major protein with molecular mass of 15 and 60 kDa, respectively (data not shown). Recombinant xTTR and the GST–xTR LBD fusion protein were stored in 10% glycerol at − 85°C for later use. The protein concentration of recombinant xTTR and the GST–xTR LBD fusion protein was determined by the dye-binding method using bovine γ-globulin as the standard (Bradford, 1976).

^{125}I-T_3 binding to TTR (TTR assay) and the TR LBD (TR assay). Recombinant xTTR (70 ng per tube) or the GST–xTR LBD fusion protein (23 ng per tube) was incubated with 0.1nM ^{125}I-T_3 in 250 μl of the buffer (20mM Tris-HCl, pH 7.5, 93mM NaCl, and 1mM $CaCl_2$ for TTR assay and 10mM Tris-HCl, pH 7.5, 1.5mM EDTA, 1mM dithiothreitol, and 10% [vol/vol] glycerol for TR assay) in the presence or absence of excess unlabeled T_3 for 1.0–1.5 h at 4°C, as described previously (Kudo and Yamauchi, 2005). Competitive ^{125}I-T_3 binding was performed with solvent alone or increasing concentrations of the unlabeled test chemical, as described previously (Yamauchi *et al.*, 2000). For the TTR assay, ^{125}I-T_3 bound to xTTR was separated from free ^{125}I-T_3 by the polyethyleneglycol method (Yamauchi *et al.*, 1993). For the TR assay, the Dowex method (Lennon, 1992; Lennon *et al.*, 1980) was used to separate ^{125}I-T_3 bound to the GST–xTR LBD protein from free ^{125}I-T_3. The radioactivity of the samples was measured in a gamma counter (Auto Well Gamma System ARC-2000, Aloka, Japan). The amount of ^{125}I-T_3 bound nonspecifically was derived from the radioactivity of those samples incubated with excess unlabeled T_3 (Ishihara *et al.*, 2003). The nonspecific binding value was subtracted from the amount of total bound ^{125}I-T_3 to give the value of specifically bound ^{125}I-T_3.

T_3-responsive reporter gene assay. Recombinant *X. laevis* XL58-TRE-Luc cells, which express high levels of luciferase in a T_3-dependent manner (Sugiyama *et al.*, 2005), were cultured in 70% Leibovitz's L-15 medium with or without 2nM T_3 and in the absence or presence of the test chemicals at various concentrations for 24 h, following incubation in 70% Leibovitz's L-15 medium containing 10% resin-stripped fetal bovine serum (FBS) (Samuels *et al.*, 1979) for 15 h. The cell lysate was assayed for firefly luciferase activity using the PicaGene Luminescence kit (Nippon Gene, Tokyo, Japan). The viability of the *X. laevis* XL58-TRE-Luc cells in the presence or absence of the test chemicals at various concentrations was photometrically examined at 450 nm using the Cell Count Reagent SF kit (Nacalai Tesque) according to the manufacturer's directions and the method described by Ishiyama *et al.* (1997).

To investigate the effect of serum proteins on the T_3 agonist and/or antagonist activity of the test chemicals in the reporter gene assay, either FBS (Invitrogen, Carlsbad, CA) or bullfrog serum was added to the 70% Leibovitz's L-15 medium to a final concentration of 10%. The bullfrog serum was prepared from *Rana catesbeiana* tadpoles in stage X (Taylor and Kollros, 1946), as described previously (Yamauchi *et al.*, 2000). In this premetamorphic stage, TTR is a major TH-binding protein in the bullfrog plasma (Yamauchi *et al.*, 1993). The 10% serum media were preincubated with or without T_3 in the presence or absence of the test chemicals for 6 h at 25°C before incubating the *X. laevis* XL58-TRE-Luc cells with the 10% serum media.

In vivo*, short-term gene expression assay.* *Xenopus laevis* tadpoles were purchased from the *Xenopus* Breeding Company (Tokyo, Japan). Tadpoles were classified according to the developmental stages outlined by Nieuwkoop and Faber (NF stages; 1994). Tadpoles were maintained in a 20-l glass aquarium containing dechlorinated tap water under natural lighting conditions and were fed dried food, which was obtained commercially and is produced for the fish medaka (Medaka-no-esa, Kyorin Co., Himeji, Japan), once a week. Before starting experiments, the tadpoles were acclimatized to laboratory conditions (20–23°C) for 24 h. During the acclimatization and exposure periods, tadpoles were not fed. Five to six tadpoles per group in NF stages 52–53 were transferred to a 1-l glass beaker containing 0.5–0.6 l of FETAX buffer (Dumont *et al.*, 1983). An *in vivo*, short-term gene expression assay was performed according to the protocol outlined previously (Kudo and Yamauchi, 2005) except that the exposure period was altered. In brief, after tadpoles were exposed to the solvent alone or the test chemicals dissolved in the solvent in the presence or absence of 2nM T_3 for 2 days, anesthetized in 0.02% 3-aminobenzoic acid ethyl ester (Sigma), and dissected into head, trunk, and tail regions. Each region was frozen separately in liquid nitrogen and then stored at − 80°C until RNA preparation. Each experiment was repeated at least twice using tadpoles from different sets of adults.

Real-time PCR. Total RNA was extracted from the frozen head and trunk regions of tadpoles using the LiCl-urea procedure (Auffray and Rougeon, 1980). The amounts of specific RNA transcripts were estimated by real-time PCR using the SYBR Green Master Mix and the ABI Prism 7000 (Applied Biosystems, Foster City, CA) after the RNA samples were treated with reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems), as described previously (Kudo and Yamauchi, 2005). Each PCR was run in triplicate to control for PCR variation. To standardize each experiment, the amount of each T_3-responsive gene transcript was divided by the amount of glyceraldehydephosphate dehydrogenase RNA in the same sample. The primer sequences used to amplify the *X. laevis* glyceraldehydephosphate dehydrogenase and TRβ transcripts were shown in a previous report (Kudo and Yamauchi, 2005). The primer sequences used to amplify the *X. laevis* TH/bZIP transcript (accession number: U41859) are sense 5′-GTTTCCGTTTTGGCCTGGTA-3′ (nucleotide numbers 413–432) and antisense 5′-CATGAAGGCCACACT-GTGTTG-3′ (487–467).

Statistical analysis. The data are presented as mean ± SEM. Differences between two groups were analyzed with Student *t* test following Bartlett's test for uniformity of variances. Data from reporter gene assay were analyzed by a one-way or a two-way ANOVA with the Fisher least significant difference test for multiple comparisons, to evaluate the significance of the differences. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Brominated Phenolic and Phenol Compounds on ^{125}I-T_3 Binding to xTTR

To determine which brominated compounds were strong competitors of ^{125}I-T_3 binding to xTTR, ^{125}I-T_3 binding to xTTR was examined in the presence of the phenolic (Fig. 1A) and phenol (Fig. 1B) compounds at various concentrations. Of bisphenol A and its brominated derivatives, 3,3′,5-tribromobisphenol A was the most powerful competitor of ^{125}I-T_3 binding to xTTR with a 50% inhibitory concentration (IC_{50}) of 1.12 ± 0.10nM. The relative affinities of 3,3′,5-tribromobisphenol A, 3,3′-dibromobisphenol A (IC_{50} = 1.80 ± 0.69nM), and 3,3′,5,5′-tetrabromobisphenol A (IC_{50} = 3.07 ± 0.29nM) for xTTR were three orders of magnitude greater than that of bisphenol A (IC_{50} = 1670 ± 30nM) and were also greater than that of T_3 (IC_{50} = 6.46 ± 0.03nM). The relative affinity of 3,3′,5-tribromobisphenol A for xTTR was twofold greater than that of 3,3′,5-trichlorobisphenol A (IC_{50} = 2.22 ± 0.09nM), which was previously identified as a potent competitor of ^{125}I-T_3 binding to xTTR (Kudo and Yamauchi, 2005). Of the bromophenols tested, pentabromophenol was the most potent competitor with an IC_{50} of 363 ± 32nM, but the relative affinity of this compound for xTTR was two orders of magnitude less than that of T_3.

Effect of Brominated Phenolic and Phenol Compounds on ^{125}I-T_3 Binding to the xTR LBD

All compounds tested were weak inhibitors of ^{125}I-T_3 binding to the xTR (Figs. 2A and 2B) compared with their

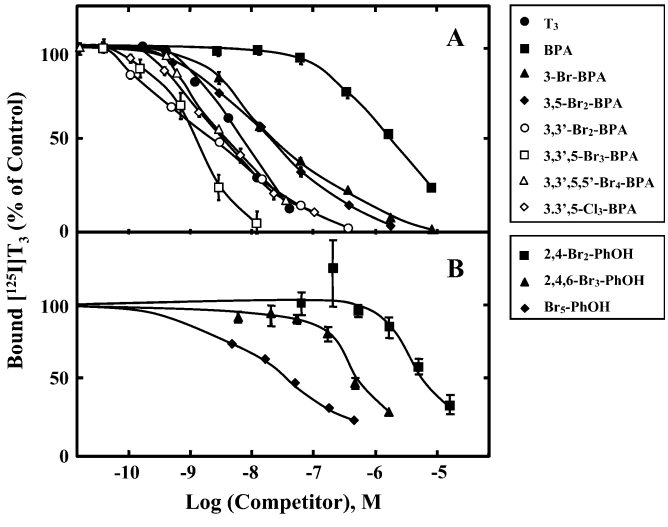

FIG. 1. Inhibition of ^{125}I-T_3 binding to purified xTTR by brominated phenolic (A) and phenol (B) compounds. Assay conditions are described in the "Materials and Methods" section. To estimate the relative potency of the test chemicals, unlabeled T_3 was examined under the same conditions. Nonspecific binding, which was less than 10% of total binding, was subtracted from total binding to give specific binding. All experiments were performed in triplicate. Deviations less than the size of the symbols are not shown. Each experiment was repeated three to four times. Abbreviations: BPA, bisphenol A; 3-Br-BPA, 3-bromobisphenol A; 3,5-Br_2-BPA, 3,5-dibromobisphenol A; 3,3′-Br_2-BPA, 3,3′-dibromobisphenol A; 3,3′,5-Br_3-BPA, 3,3′,5-tribromobisphenol A; 3,3′,5,5′-Br_4-BPA, 3,3′,5,5′-tetrabromobisphenol A; 3,3′,5 -Cl3-BPA, 3,3′,5, -trichlorobisphenol A; 2,4-Br_2-PhOH, 2,4-dibromophenol; 2,4,6-Br_3-PhOH, 2,4,6-tribromophenol; Br_5-PhOH, pentabromophenol.

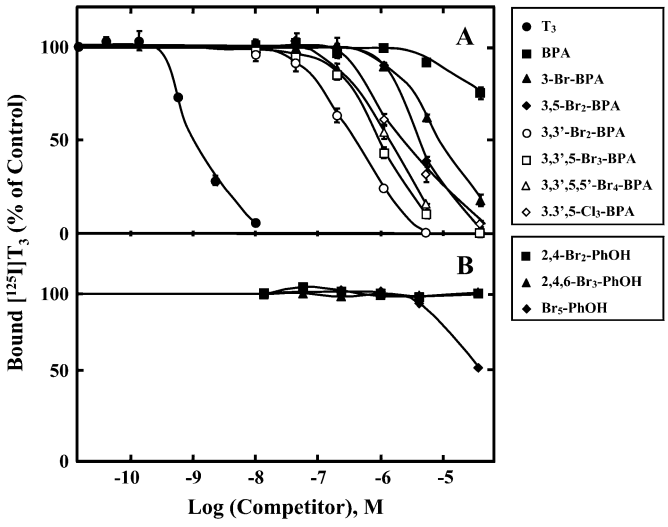

FIG. 2. Inhibition of ^{125}I-T_3 binding to the GST-fused, ligand-binding domain of *Xenopus laevis* TR (GST-xTR LBD) by brominated phenolic (A) and phenol (B) compounds. Assay conditions are described in the "Materials and Methods" section. To estimate the relative potency of the test chemicals, unlabeled T_3 were examined under the same conditions. Nonspecific binding, which was less than 10% of total binding, was subtracted from total binding to give specific binding. All experiments were performed in triplicate. Deviations less than the size of the symbols are not shown. For abbreviations see the legend of Figure 1.

effect on ^{125}I-T_3 binding to xTTR (Fig. 1). The compounds with an IC_{50} of less than 10^{-6}M were 3,3′-dibromobisphenol A (360 ± 50nM) and 3,3′,5-tribromobisphenol A (790 ± 10nM). The relative affinities of the two compounds for xTR were still several hundred fold less than that of T_3 (IC_{50} = 1.33 ± 0.02nM). The relative affinity of 3,3′,5-tribromobisphenol A for xTTR was threefold greater than that of 3,3′,5-trichlorobisphenol A (IC_{50} = 2220 ± 90nM). The rank order–binding affinities of the brominated phenolic and phenol compounds for xTR were similar to those for xTTR.

Effect of Brominated Phenolic and Phenol Compounds on T_3-Responsive Reporter Gene Assay in a Recombinant X. laevis *Cell Line*

We investigated the effect of the brominated phenolic and phenol compounds on a T_3-responsive reporter gene assay in the recombinant *X. laevis* cell line, XL58-TRE-Luc. In the absence of the brominated phenolic and phenol compounds, T_3 (2nM) increased the luciferase activity by about threefold. Cotreatment of 3,3′-dibromobisphenol A, 3,3′,5-tribromobisphenol A, or 3,3′,5,5′-tetrabromobisphenol A with T_3 significantly inhibited the T_3-induced luciferase activity in a dose-dependent manner, demonstrating their T_3 antagonist activity (Fig. 3A). The mean inhibition percentage obtained from independent repeated experiments (n = 3) was 85 ± 5% for 0.1μM 3,3′-dibromobisphenol A ($p < 0.05$ compared with control), 67 ± 9% for 0.1μM 3,3′,5-tribromobisphenol A ($p < 0.01$), and 67 ± 8% for 0.1μM 3,3′,5,5′-tetrabromobisphenol A ($p < 0.05$). The effect of other compounds tested on the luciferase activity was small or not significant although 3-bromobisphenol A and 3,5-dibromobisphenol A had a tendency to inhibit the T_3-induced luciferase activity in a dose-dependent manner. When the cells were exposed to these compounds in the absence of T_3 (Fig. 3B), the luciferase activity increased significantly by 121 and 129% in the presence of 3,3′,5-tribromobisphenol A ($p < 0.05$ at 0.01μM; $p < 0.01$ at 0.1 and 1.0μM) and 3,3′,5,5′-tetrabromobisphenol A ($p < 0.01$ at 0.1 and 1.0μM), respectively, demonstrating that 3,3′,5-tribromobisphenol A and 3,3′,5,5′-tetrabromobisphenol A had T_3 agonist activity.

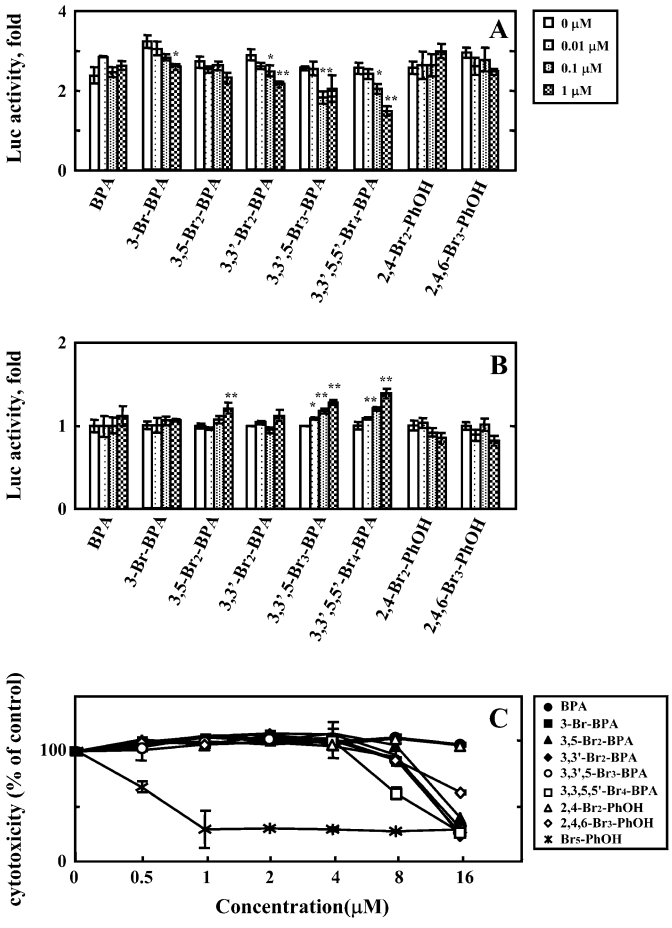

FIG. 3. Effect of brominated phenolic and phenol compounds on T_3-responsive reporter gene assay. XL58-TRE-Luc cells with T_3-responsive luciferase gene were treated with (A) or without 2nM T_3 (B), in the presence or absence (control) of various concentrations (10^{-8}–10^{-6}M) of each chemical, in serum-free 70% Leibovitz's L-15 medium. The vertical axes represent the luciferase activity as a magnitude of the induction (fold induction). Viability of the same cells (C) was investigated following the incubation with or without (control) various concentrations of each chemical for 48 h, as described in the "Materials and Methods" section. Experiments for each chemical were repeated at least three times. Each value is the mean ± SEM (n = 3). Statistically significant differences were determined by a one-way ANOVA with the Fisher least significant difference test for multiple comparisons (*$p <$ 0.05 and **$p < 0.01$ compared with control). For abbreviations of the chemicals used see the legend of Figure 1.

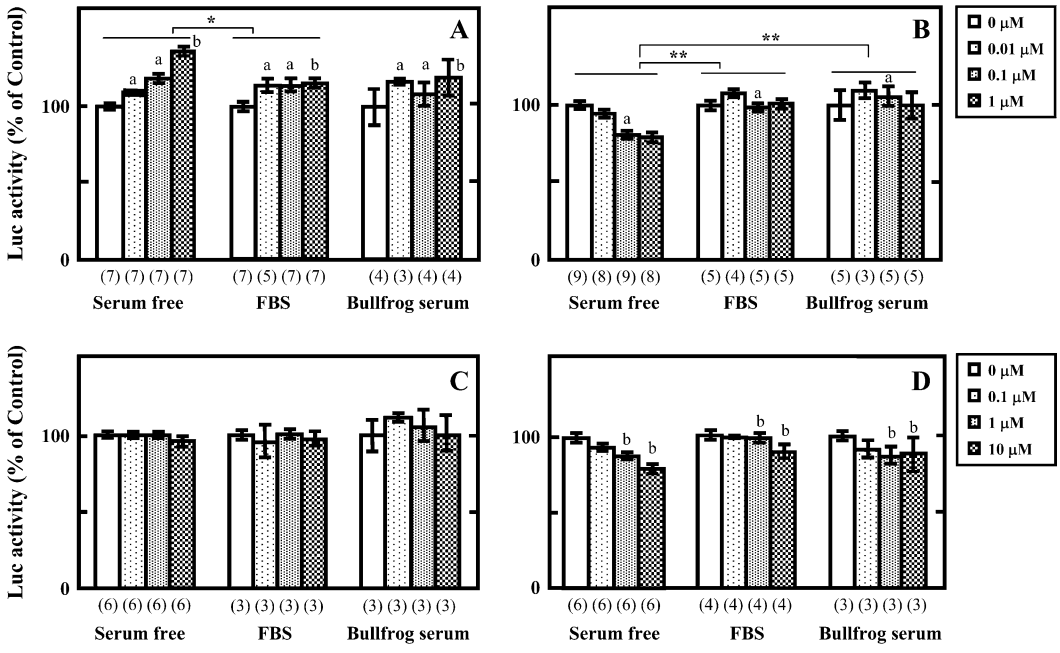

FIG. 4. Effect of sera on T_3 agonist and antagonist activities of 3,3′,5-tribromobisphenol A and *o-t*-butylphenol examined in the T_3-responsive reporter gene assay. XL58-TRE-Luc cells were treated with solvent alone (A, C) and 2.0nM T_3 (B, D), in the presence or absence (control) of various concentrations of 3,3′,5-tribromobisphenol A (10^{-8}–10^{-6}M; A, B) and *o-t*-butylphenol (10^{-7}–10^{-5}M; C, D). These experiments were performed in serum-free 70% Leibovitz's L-15 medium (left in each panel), 70% Leibovitz's L-15 medium containing 10% FBS (middle in each panel), and 70% Leibovitz's L-15 medium containing 10% bullfrog tadpole serum (right in each panel). The vertical axes represent the luciferase activity as a magnitude of the induction (% of control). The numbers of samples in each group are shown in parentheses. Data were analyzed by a two-way ANOVA with the Fisher least significant difference test for multiple comparisons to evaluate the effects of two factors: dose (a $p < 0.05$ and b $p < 0.01$ compared with dose control) and serum (*$p < 0.05$ and **$p < 0.01$ compared with serum control).

Other compounds had little or no effect on the luciferase activity even though 3,5-dibromobisphenol A and 3,3′-dibromobisphenol A had a tendency to increase the luciferase activity in a dose-dependent manner. Cell viability was not compromised in the presence of all chemicals (0.5–4μM) tested except for pentabromophenol, which was cytotoxic at 0.5μM (Fig. 3C).

Effect of Serum Proteins on T_3 Agonist and Antagonist Activities of the Compounds in a Recombinant X. laevis *Cell Line*

To determine the extent with which serum proteins affected T_3 agonist and antagonist activities of the brominated phenolic and phenol compounds in XL58-TRE-Luc cells, we conducted the reporter gene assay in the absence or presence of 10% serum (FBS and bullfrog tadpole serum) and examined the T_3 agonist and antagonist activities of 3,3′,5-tribromobisphenol A (Figs. 4A and 4B) and *o-t*-butylphenol (Figs. 4C and 4D). In the 3,3′,5-tribromobisphenol A treatment, dose effects on T_3 agonist (Fig. 4A) and antagonist (Fig. 4B) activities were significant compared with control ($p < 0.05$ at 0.1μM). Serum effects on T_3 agonist (Fig. 4A) and antagonist (Fig. 4B) activities were significant in FBS ($p < 0.05$) and in both FBS and bullfrog serum ($p < 0.01$), respectively. In the *o-t*-butylphenol treatment, T_3 agonist activity was not detected (Fig. 4C), but T_3 antagonist activity was detected (Fig. 4D). Dose effect on T_3 antagonist activity (Fig. 4D) was significant ($p < 0.01$ at 1.0 and 10μM); however, no serum effect on T_3 antagonist activity (Fig. 4D) was detected. These results demonstrated that the serum proteins in the culture medium weakened both T_3 agonist and antagonist activities of 3,3′,5-tribromobisphenol A but did not influence the T_3 antagonist activity of *o-t*-butylphenol.

Effect of 3,3′,5-Tribromobisphenol A on Short-Term Gene Expression in X. laevis *Premetamorphic Tadpoles*

In the trunk and head regions of *X. laevis* tadpoles treated with T_3 (2nM), the amount of the TRβ gene transcript increased 23- and 9-fold, respectively, whereas the amount of the TH/bZIP gene transcript increased 42- and 13-fold, respectively, on the second day after treatment (Figs. 5B and 5D). Treatment of the tadpoles with 0.5μM 3,3′,5-tribromobisphenol A in the absence of T_3 significantly increased the amounts of the TRβ and TH/bZIP gene transcripts by 200% ($p < 0.05$) and 198% ($p < 0.05$) of the control, respectively, in the trunk (Fig. 5A), but not in the head where they were 122 and 95% of the control, respectively (Fig. 5C). In the trunk of tadpoles cotreated with 0.5μM 3,3′,5-tribromobisphenol A and T_3, the T_3-induced increase in the amount of the TH/bZIP gene transcript was significantly inhibited and was 61% of the control ($p < 0.05$), whereas the T_3-induced increase in the amount

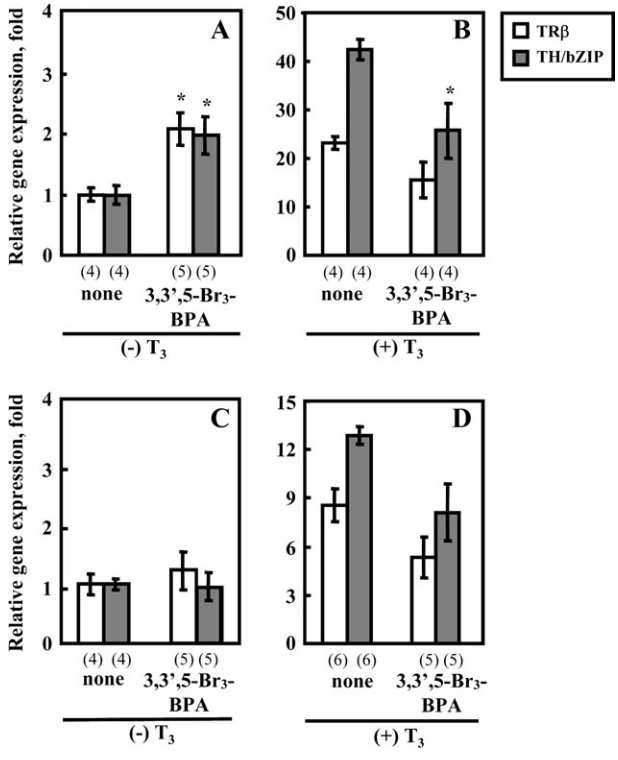

FIG. 5. Effect of 3,3′,5-tribromobisphenol A on the amounts of primary T_3-responsive gene transcripts in *Xenopus laevis* tadpoles. Total RNA was extracted from the trunk (A and B) and head (C and D) regions of spontaneous (A and C) and T_3-induced (B and D) metamorphosing tadpoles after 2 days of exposure to: solvent alone as a negative control, 2nM T_3 as a positive control, 0.5μM 3,3′,5-tribromobisphenol A (3,3′,5-Br_3-BPA) alone, and 2nM T_3 with 0.5μM 3,3′,5-tribromobisphenol A (3,3′,5-Br_3-BPA). The vertical axes represent the amounts of T_3-responsive gene transcripts for TRβ and TH/bZIP as a magnitude of the induction (fold induction). Each value is the mean ± SEM. The numbers of samples in each group are shown in parentheses. Statistically significant differences from negative (− T_3; A and C) or positive (+ T_3; B and D) control were determined by Student *t* test following Bartlett's test for uniformity of variances (*$p < 0.05$).

of the TRβ gene transcript was not significantly inhibited and was 67% of the control (Fig. 5B). In the head of tadpoles, cotreatment of 0.5μM 3,3′,5-tribromobisphenol A with T_3 did not significantly affect the T_3-induced increase in the amount of the two transcripts, which were 62–63% of the control (Fig. 5D). These results demonstrated that 3,3′,5-tribromobisphenol A elicited T_3 agonist and antagonist activities in at least the trunk region of *X. laevis* premetamorphic tadpoles.

DISCUSSION

The present study demonstrated that 3,3′,5-tribromobisphenol A exhibited both T_3 agonist and antagonist activities at submicromolar concentrations in a *X. laevis* cell line and *X. laevis* tadpoles. This compound competed with T_3 binding to xTTR and xTR *in vitro*, suggesting that xTTR and xTR are possible target sites for 3,3′,5-tribromobisphenol A. These results raised the possibility that the flame retardant 3,3′,5-tribromobisphenol A interferes with extracellular TH homeostasis by binding TTR, which is a major TH-binding protein in amphibian tadpoles (Yamauchi *et al.*, 1993), and with the intracellular TH-signaling pathway by binding TR.

Our previous studies on the competitive inhibition of ^{125}I-T_3 binding to TTRs and TRs revealed a structure-activity relationship between the chlorinated compounds and THs (Kudo and Yamauchi, 2005; Yamauchi *et al.*, 2003). These studies revealed that (1) generally, the relative potency of the chlorinated phenolic and phenol compounds with which they interfered with ^{125}I-T_3 binding to TTRs and TRs was dependent upon the degree of chlorination of the compound; (2) TRs from amphibians and chicken and TTRs from amphibians preferentially bind compounds with structures that are similar to T_3, such as chlorinated phenolic compounds that have two phenolic rings with chlorines in either *ortho* positions with respect to the hydroxy group, e.g., 3,3′-dichlorobisphenol A and 3,3′,5-trichlorobisphenol A, whereas TTR from chicken preferentially binds phenolic compounds with structures that are similar to T_4, such as chlorinated phenolic compounds that have two phenolic rings with chlorines in both *ortho* positions with respect to the hydroxy group, e.g., 3,3′,5,5′-tetrachlorobisphenol A; and (3) the affinity of the chlorinated phenolic and phenol compounds for TRs were three orders of magnitude less than that of T_3, whereas the affinity of the chlorinated phenolic and phenol compounds for TTRs were comparable with that of T_3. Thus, it was concluded that the binding preferences of TTR and TR for particular chlorinated phenolic compounds may reflect the TH-binding properties of TTR and TR and the structural resemblance between the chlorinated phenolic compounds and THs. This conclusion is particularly relevant given that mammalian TTRs have a four- to eightfold higher affinity for T_4 than T_3 (Chang *et al.*, 1999; Robbins, 1996), whereas xTTR and the bullfrog TTR have a 100- to 400-fold higher affinity for T_3 than T_4 (Yamauchi *et al.*, 1993, 2002). The structure-activity relationship identified for the chlorinated compounds and THs may also be true for the binding of the brominated compounds to *X. laevis* and human TTRs (this study; Meerts *et al.*, 2000) and TRs (this study; Kitamura *et al.*, 2002). In addition to these studies, the present and previous studies (Kudo and Yamauchi, 2005) found that xTTR, like human TTR (Meerts *et al.*, 2000), had several times higher affinity for the brominated derivatives of bisphenol A than for the chlorinated derivatives of bisphenol A, whereas xTTR, unlike human TTR (Meerts *et al.*, 2000), had several times lower affinity for the bromophenols than for the chlorophenols. These binding characteristics suggest that generally a structure-activity relationship exists between the structurally related, halogenated compounds and THs. It should be noted that there are species differences in TH-binding proteins. TTR is a relatively minor binding protein in human plasma but a major binding protein in amphibian plasma (Schreiber and Richardson, 1997). This raises the possibility that some halogenated

compounds affect plasma TH homeostasis in a species-specific manner by binding TTR.

The T_3-responsive reporter gene assay using the recombinant *X. laevis* cell line XL58-TRE-Luc revealed T_3 agonist and antagonist activities of both 3,3′,5-tribromobisphenol A and 3,3′,5,5′-tetrabromobisphenol A at 10^{-7}–10^{-6}M. The effective concentrations of 3,3′,5-tribromobisphenol A and 3,3′,5,5′-tetrabromobisphenol A in the T_3-responsive reporter gene assay corresponded well to those recorded for the inhibition of ^{125}I-T_3 binding to xTR. Therefore, it is highly likely that these compounds exerted T_3 agonist and antagonist activities by binding to xTR. Recently, it has been reported that some lipophilic chemicals accumulate into cultured cells by one to two orders of magnitude in tissue-, time-, and concentration-dependent manners (Maecham *et al.*, 2005). It is likely that the lipophilicity, cellular influx or efflux by specific transporters, metabolism of a compound (Wong *et al.*, 2005) and the presence of high-affinity binding sites other than the T_3-binding site of the TR for a compound would affect its intracellular free concentration or its action through the TR-coregulator complex. Our findings do not exclude the possibility that 3,3′,5-tribromobisphenol A and 3,3′,5,5′-tetrabromobisphenol A target an intracellular process other than T_3 binding to the TR that disrupts the thyroid system. Recent studies have demonstrated that bisphenol A interfered with the thyroid system by recruiting the nuclear corepressor to the human TR (Moriyama *et al.*, 2002) and that some hydroxylated polychlorinated biphenyls caused the partial dissociation of the TR/retinoid X receptor heterodimer complex from the TH-responsive elements (Miyazaki *et al.*, 2004). The concentrations of bisphenol A (0.1–1.0μM) and hydroxylated polychlorinated biphenyls (0.1pM) required to elicit T_3 antagonist activity were several orders of magnitude lower than their IC_{50} values for competitive binding to the human TR (Cheek *et al.*, 1999; Moriyama *et al.*, 2002). This indicated that bisphenol A and the hydroxylated polychlorinated biphenyls affected TR activation without displacing T_3 from the TR. A recent review (Tabb and Blumberg, 2006) proposed several novel modes by which environmental chemicals could disrupt the steroid system that included the modulation of the proteasome-mediated degradation of nuclear receptors and their coregulators, inhibition of histone deacetylase activity, and stimulation of mitogen-activated protein kinase activity. In the thyroid system, it remains to be clarified whether environment chemicals disrupt processes of gene transcription other than T_3 binding to the TRs.

In our reporter gene assay using an *in vitro* cell culture system, we found that serum proteins were capable of weakening T_3 agonist and antagonist activities of 3,3′,5-tribromobisphenol A. However, serum proteins did not influence T_3 antagonist activity of *o-t*-butylphenol. Considering the fact that 3,3′,5-tribromobisphenol A had an affinity that was three orders of magnitude higher for xTTR than that of *o-t*-butylphenol (Fig. 1 and Kudo and Yamauchi, 2005), TTR may selectively influence the cellular action of the thyroid system–disrupting chemicals depending upon its affinity for chemicals.

Our *in vivo*, short-term gene expression assay in tadpoles confirmed that 3,3′,5-tribromobisphenol A elicited T_3 agonist and antagonist activities. As the TH/bZIP gene was induced with greater extent than the THβ gene by treatment with T_3 in the trunk and head, the TH/bZIP gene may be a suitable molecular marker for detecting thyroid system–disrupting chemicals. The amphibian metamorphosis–based assays conducted by other groups (Jagnytsch *et al.*, in press; Kitamura *et al.*, 2005) revealed T_3 antagonist and/or agonist activities of 3,3′,5,5′-tetrabromobisphenol A. However, the effect of 3,3′,5-tribromobisphenol A in our *in vivo*, short-term gene expression assay in premetamorphic tadpoles was not as clear as that found in the *in vitro* assay using the cell line (Fig. 5 vs. Figs. 3 and 4). The thyroid system–disrupting activity of 3,3′,5-tribromobisphenol A was barely detected in the *X. laevis* trunk and was not detected in the head. Therefore, the sensitivity of different tissues to THs, the endpoints investigated, the developmental stage selected (OECD, 2004), the modulation of the effect of the chemical by plasma proteins including TTR, and the variation in the response to THs among individual tadpoles (Turque *et al.*, 2005) may all be important factors for clearly detecting and identifying the thyroid system–disrupting activity of the environmental chemicals in *in vivo* studies.

The thyroid system–disrupting activity of 3,3′,5-tribromobisphenol A identified in our *in vitro* reporter gene assay and *in vivo*, short-term gene expression assay was acute. We cannot fully determine to what extent the thyroid system–disrupting activity of 3,3′,5-tribromobisphenol A is modulated by plasma *in vivo* and what impact this chemical has on the amphibian thyroid system after long-term exposure. The *in vivo* situation in tadpoles after their long-term exposure to environmental chemicals for which TTR has a high affinity may be close to the situation found in wildlife chronically exposed to halogenated persistants, such as the polychlorinated biphenyls. A negative correlation between polychlorinated biphenyls and TH levels has been found in the plasma of seals (Chiba *et al.*, 2001) and polar bears (Braathen *et al.*, 2004). However, data are very limited regarding the presence of brominated derivatives of bisphenol A in biota. Serum levels of tetrabromobisphenol A ranged from 1 to 3.4 pmol/g lipid in human (Jakobsson *et al.*, 2002). In tadpoles, the dynamic destination of 3,3′,5-tribromobisphenol A, the half-life of TTR *in vivo*, the expression level of TTR, and the plasma levels of THs after exposure to 3,3′,5-tribromobisphenol A remains to be clarified.

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