Suppression of Amphibian Metamorphosis by Bisphenol A and Related Chemical Substances

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This investigation attempts to clarify the effects of the plastic monomer bisphenol A (BPA) and related chemicals on 3,3′,5-triiodothyronine (T_3)-induced and spontaneous anuran tadpole tail regression. T_3-induced tail regression was found to be suppressed by BPA and tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA), and tetramethylbisphenol A (TMBPA). T_3-treated *Rana rugosa* tadpole tails displayed marked apoptotic features, including DNA fragmentation and ladder-like profiles, as opposed to essentially little or no fragmentation and ladder formation for BPA, TBBPA, TCBPA and TMBPA-treated tails. BPA and related compounds also inhibited *Silurana tropicalis* spontaneous metamorphosis controlled by endogenous circulating thyroid hormone (TH). These results indicate that BPA and related compounds are TH antagonists. In transgenic *Xenopus laevis* tadpoles carrying plasmid DNA containing TH response element (TRE) and 5′-upstream promoter region of the TH receptor (TR) βA1 gene linked to a green fluorescent protein (EGFP) gene, T_3 induced a strong EGFP expression in the hind limbs, while T_3 plus BPA, TBBPA, TCBPA or TMBPA suppressed the expression, suggesting BPA and related chemicals all act in preventing the binding of T_3 to TR, resulting in inhibition of TR-mediated gene expression.

Key words ——— amphibian metamorphosis, apoptosis, bisphenol A related compound, endocrine disruptor, thyroid hormone antagonist, transgenic frog

INTRODUCTION

Endocrine disruptors include a group of chemicals which disturb normal endocrine activity, and are a suspected cause in the depletion of wildlife populations and various human health problems.[1–4] Bisphenol A (BPA) — an industrial raw material for polycarbonate and epoxy resins — is one of the most common endocrine disruptors. BPA has been found to antagonize thyroid hormone (TH) action *in vitro* and *in vivo* assays.[5,6] The brominated BPA analogue, tetrabromobisphenol A (TBBPA), is widely used as a flame retardant in building materials, paints, *etc.*,[7] and has been reported to be weakly estrogenic *in vitro*,[8–10] to bind thyroid hormone receptor (TR) *in vitro*,[11,12] and to antagonize the biological function of TH *in vivo*.[12] TBBPA inhibits larval development in *Xenopus laevis* (*X. laevis*).[13] The chlorinated structural analogue, tetrachlorobisphenol A (TCBPA), is found in the effluent from waste-paper recycling plants.[14] The methylated structural analogue, tetramethylbisphenol A (TMBPA), is used in polycarbonate resin. Both TCBPA and TMBPA have been found to bind the TR.[11,12]

TH is important in various physiological processes in animal metabolism, development and growth. Amphibian tadpole metamorphosis is a TH-controlled developmental process.[15–19] TR forms a heterodimer with a retinoid X receptor (RXR), thereby binding to the thyroid hormone response element (TRE) in the target genes. This is followed by binding to 3,3′,5-triiodothyronine (T_3) to enhance or repress gene transcription.[18] 3,3′,5,5′-tetraiodothyronine (T_4) regulates the expression of a va-

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riety of genes.[20] Of the genes which are regulated by THs in the tail shortening program of *X. laevis*, 17 genes have been observed to be up-regulated and 4 genes down-regulated.[16] Tadpole tail regression during anuran metamorphosis is the result of muscle cell apoptosis, or programmed cell death,[19,21,22] and is triggered and controlled by THs. Apoptosis hallmarks include chromatin condensation, protease and endonuclease activation, internucleosomal DNA fragmentation, ladder formation, membrane blebbing and cell shrinkage.[23] Little information is available regarding the effects of BPA and related compounds on the amphibian thyroid system and metamorphosis. The addition of the THs T_3 and T_4 to the culture medium (water) of premetamorphic stage tadpoles results in an enhancement of the metamorphic process,[24–26] and this TH-induced enhancement has proven to be a useful tool for investigating the effects of various substances [for example *N*-monomethyl-L-arginine (NMMA), *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), cyclosporin A] on the thyroid system and amphibian growth and development.[22,27] In the present investigation, we use TH-induced enhancement to examine the disruptive effects of BPA, TBBPA, TCBPA and TMBPA on T_3 activity (Fig. 1) and tail cell apoptosis in three amphibian species. We also examine their effect on TR-mediated gene expression.

MATERIALS AND METHODS

Chemicals —— BPA, TBBPA, TCBPA and TMBPA were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). T_3 and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Methimazole was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). Test compounds were solubilized in DMSO, and assays were conducted in 0.1% DMSO solution.

Animal Maintenance —— Three amphibian species, *Rana rugosa* (*R. rugosa*), *Silurana tropicalis* (*S. tropicalis*) and *X. laevis* were used in the present investigation.

Adult specimens of *R. rugosa* were raised in the laboratory. Ovulation was induced by injecting *Rana catesbeiana* (bullfrog) pituitaries into the body cavity of mature females. Eggs from a single female were artificially fertilized with a chlorine-free water suspension of crushing male testes. Tadpoles were
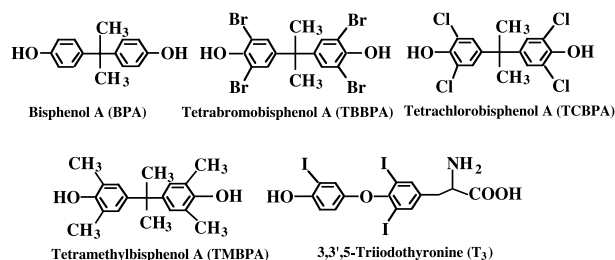

Fig. 1. Structures of BPA, TBBPA, TCBPA, TMBPA and T_3

fed on boiled spinach and staged according to the criteria of Taylor and Kollros.[28]

S. tropicalis used in the present study were maintained in the Institute for Amphibian Biology, Graduate School of Science, Hiroshima University as part of a national bioresource project conducted by the Ministry of Education, Culture, Sports, Science and Technology, while adult *X. laevis* were derived from standard strains maintained by the Hiroshima University Institute for Amphibian Biology. Mating for both species was induced by injecting human chorionic gonadotropin (hCG; Sigma) into the dorsal lymph sac (*S. tropicalis* males — 125 U, females — 300 U; *X. laevis* males — 125 U, females — 500–600 U). Matings for *S. tropicalis* were carried out by placing one male-female pair into a tank with 5–10 cm of chlorine free tap water at 23°C for 4–5 hr, and matings for *X. laevis* were carried out at 19°C for 10–12 hr. Tadpoles of both species were raised with SERA Micron (Sera Heinsberg, Germany) and staged according to Nieuwkoop and Faber.[29]

Extraction of DNA and Analysis of Fragmentation and Laddering —— DNA was extracted from the tail using phenol/chloroform (1 : 1) as described previously.[30,31] Tails were homogenized in a lysis buffer (50 mM Tris–HCl, pH 7.8, 10 mM EDTA, 0.5% sodium-*N*-lauroylsarcosinate). Fragmentation of DNA was quantified by the diphenylamine method.[30] Briefly, the lysate was centrifuged at $13000 \times g$ at 4°C for 20 min to separate intact and fragmented chromatin. Each fraction was treated with 0.2 ml of 6% perchloric acid (PCA) for 30 min and then centrifuged at $13000 \times g$ for 20 min at 4°C. Precipitated fractions were treated with 50 μl of 6% PCA at 70°C for 20 min. 0.1 ml of diphenylamine solution (1.5% diphenylamine, 1.5% sulfuric acid, 0.01% acetaldehyde in acetic acid) was added to the samples and these were allowed to stand overnight at 30°C. The samples were then determined spectrophotometrically at 600 nm, and the percentage of

fragmented DNA was calculated from the amount of DNA found in the supernatant.

Ladder-like profiles of electrophoresed DNA samples were analyzed as described previously.[31] Briefly, homogenates were incubated with 1 mg/ml of proteinase K for 30 min and subsequently with 1 mg/ml of RNase for 30 min at 50°C. The DNA samples thus obtained (1.5 from 5 μg of total DNA) were subjected to 2% agarose gel electrophoresis at 100 V using a running buffer containing 90 mM Tris–HCl (pH 8.0), 90 mM boric acid and 2 mM EDTA. After electrophoresis, gels were stained with 0.1 μg/ml ethidium bromide and observed under ultraviolet light.[32]

Assay of Enhanced Green Fluorescent Protein (EGFP) Activity —— Transgenic *X. laevis* were produced using the transgenesis procedure described in Kroll and Amaya,[33] by which plasmid DNA containing 5′-upstream promoter region plus TRE of the TRβA1 gene linked to EGFP gene is integrated into the chromosomes of isolated sperm nuclei *in vitro*, followed by transplantation of the nuclei into unfertilized eggs to begin development, producing transgenic tadpoles and frogs (F_0).[34] In the resulting transgenic embryos and tadpoles, a weak EGFP expression was first observed at the neurula stage, and a strong expression was observed at metamorphic climax. In the present study, mature first generation offspring (F_1) males were mated with normal females. All transgenic males produced offspring (second generation offspring, F_2) in large numbers, and T_3-treated offspring showed stable EGFP expression ubiquitously throughout the whole body, particularly strongly in the hind limbs.

EGFP expression in tadpoles was monitored using a fluorescent dissecting microscope (MZ FLIII, Leica) equipped with a digital camera (D70, Nikon, Tokyo, Japan). Quantitative analysis of EGFP expression was performed using Corel PHOTO-PAINT 11 (Corel Corporation, Ontario, Canada).

Statistical Analyses —— DNA fragmentation was analyzed using the Chi-square test. Unless otherwise stated, results are expressed as the mean ± SEM. Statistical differences between untreated control and BPA and related compounds or T_3-treated groups were analyzed using Student's *t*-test and Welch's *t*-test. *p*-Values below 0.05 are considered significant.
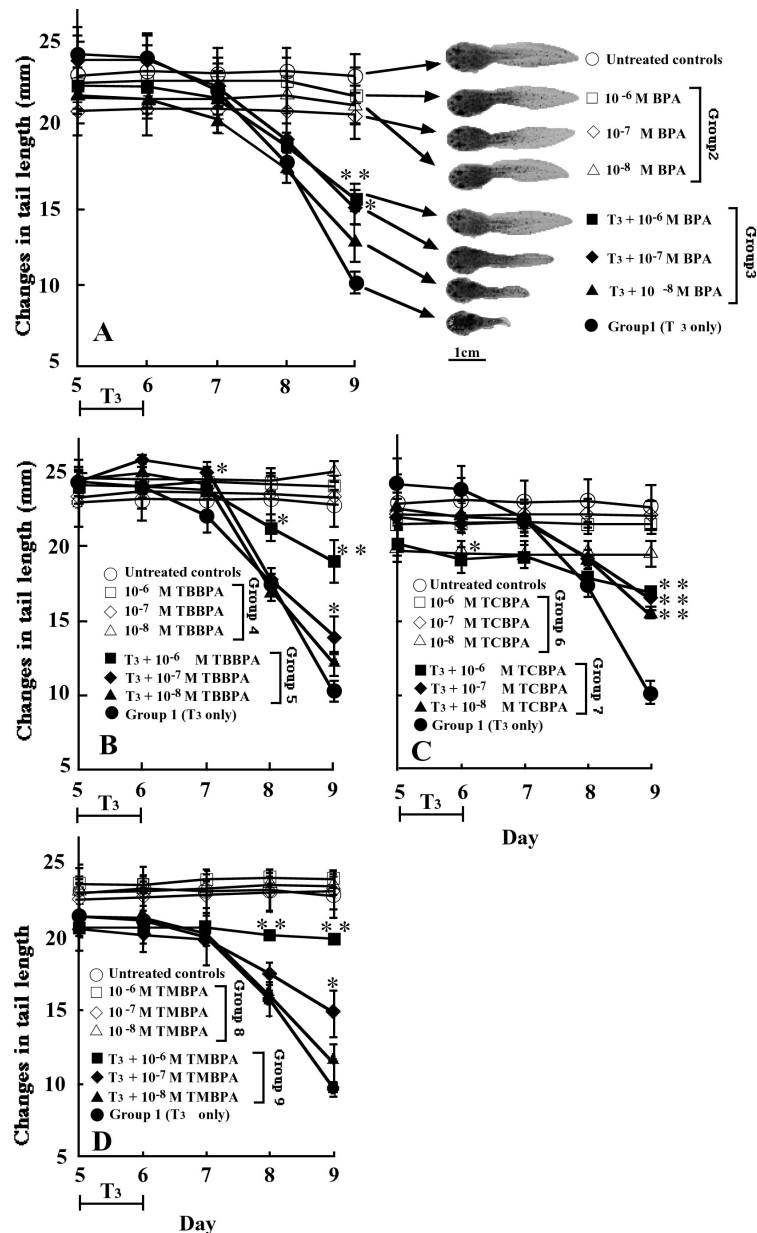
RESULTS

Effect of BPA and Related Compounds on Tadpole Tail Shortening in T_3-Induced Metamorphosis

Figure 2 shows the effect of BPA, TBBPA, TCBPA and TMBPA on tail shortening in T_3-treated and untreated tadpoles. Stage X *R. rugosa* tadpoles were raised at a population density of one individual per 50 ml in trays containing various solutions as follows: Group-1 tadpoles in Cl-free tap water, group-2 and -3 tadpoles in tap water containing 10^{-6} to 10^{-8} M of BPA, group-4 and -5 tadpoles were raised in tap water containing 10^{-6} to 10^{-8} M TBBPA, group-6 and -7 tadpoles were raised in tap water containing 10^{-6} to 10^{-8} M TCBPA, and group-8 and -9 tadpoles were raised in tap water containing 10^{-6} to 10^{-8} M TMBPA. After 5 days, 5×10^{-8} M of T_3 was added to the water of groups 1, 3, 5, 7 and 9, and treatment was continued for one day. At the end of T_3 treatment, group-1 tadpoles were returned to water only, group-3 tadpoles to the BPA solution, group-5 tadpoles to the TBBPA solution, group-7 tadpoles to the TCBPA solution, and group-9 tadpoles to the TMBPA solution, and these groups were further maintained for another 3 days. Groups 2, 4, 6, and 8 continued treatment for all 9 days with no addition of T_3. Control tadpoles were raised in water and not exposed to BPA, TBBPA, TCBPA, TMBPA or T_3. The results show that T_3 induces tail shortening, and that this is suppressed by BPA (10^{-6} and 10^{-7} M), TBBPA (10^{-6} and 10^{-7} M), TCBPA (10^{-6} to 10^{-8} M) and TMBPA (10^{-6} and 10^{-7} M) in a dose–dependent manner.

Effects of BPA and Related Compounds on T_3-Induced Tail Apoptosis

Figure 3 shows the effect of BPA, TBBPA, TCBPA and TMBPA on DNA fragmentation and ladder formation in tails of T_3-treated and untreated *R. rugosa* tadpoles. Experimental conditions were the same as described above. DNA fragmentation and ladder formation were carried out on the third day after completion of T_3 treatment. DNA in tails of T_3-treated group-1 tadpoles showed marked fragmentation (Fig. 3A and 3C) and a ladder-like profile (Fig. 3B and 3D). These changes were not observed in group-3 ($T_3 + 10^{-6}$ M BPA), group-5 ($T_3 + 10^{-6}$ M TBBPA), group-7 ($T_3 + 10^{-6}$ M TCBPA) or group-9 ($T_3 + 10^{-6}$ M TMBPA) tadpoles. Nor were such changes observed in group-2 (10^{-6} M BPA),
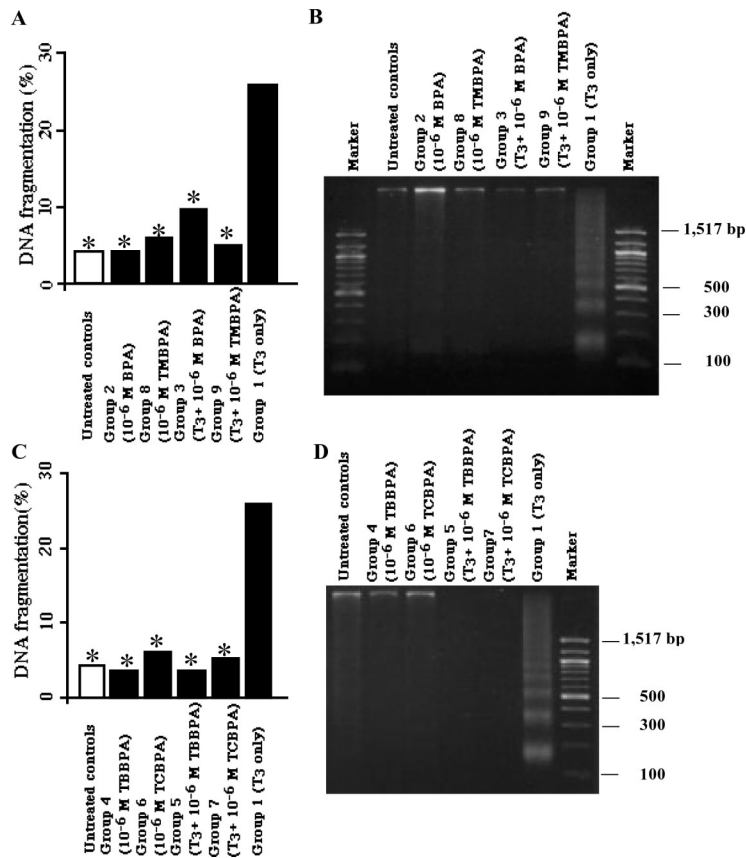
Fig. 2. Effect of BPA, TBBPA, TCBPA and TMBPA on Tail Shortening in T_3-Treated and Untreated *R. rugosa* Tadpoles
*Significantly greater ($p < 0.05$) than corresponding values for group-1 tadpoles. **Significantly greater ($p < 0.01$) than corresponding values for group-1 tadpoles.

group-4 (10^{-6} M TBBPA), group-6 (10^{-6} M TCBPA) or group-8 (10^{-6} M TMBPA) tadpoles.

Effects of BPA and Related Compounds on Spontaneous Metamorphosis, Tail Shortening and Hindlimb Elongation

S. tropicalis is becoming increasingly popular for use in developmental and genetic studies because it possesses a diploid genome[35–37] and has a relatively short generation time of approximately 4 months for males and 5 months for females. In the

species *X. laevis* endogenous plasma TH values are low at prometamorphic stages 56–57,[38] but increase markedly at midclimax stages 61–62[38] at the beginning of spontaneous rapid tadpole tail shortening.[39] Endogenous plasma TH values are not available for *S. tropicalis*, but we assume they operate in a manner similar to that seen in *X. laevis*. Two stage 57 *S. tropicalis* tadpoles were kept in trays containing 200 ml of 10^{-6} M solutions of BPA, TBBPA, TCBPA or TMBPA, or a 1 mM solution of the methimazole TH synthesis inhibitor. Tadpoles raised in Cl-free

Fig. 3. Effect of BPA, TBBPA, TCBPA and TMBPA on DNA Fragmentation and Ladder Formation in Tails of T_3-Treated and Untreated *R. rugosa* Tadpoles

(A, C) DNA fragmentation; (B, D) Ladder formation. Each experiment was carried out using three tadpoles. Values given represent the mean value of three repetitions. *Significantly less ($p < 0.01$) than group-1 value.
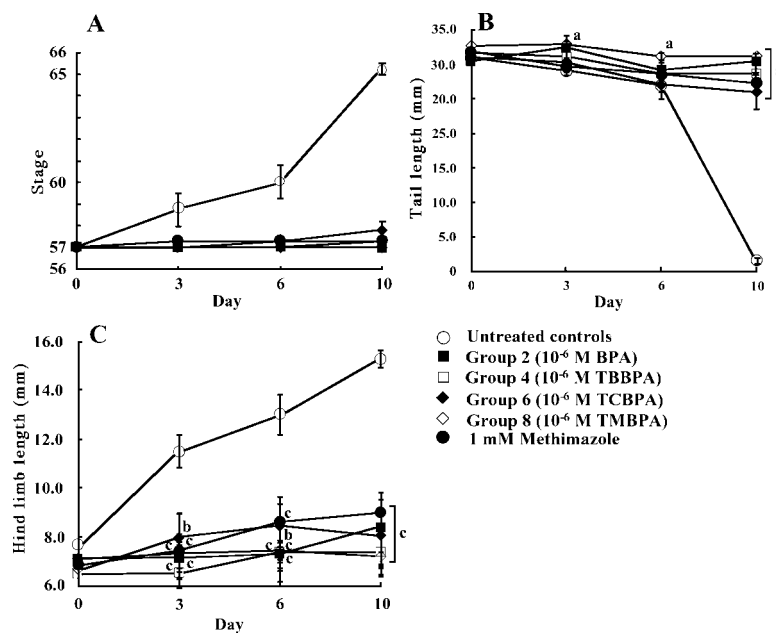
Fig. 4. Suppression of Spontaneous Metamorphosis (A), Tadpole Tail Shortening (B) and Hindlimb Elongation (C) by BPA, TBBPA, TCBPA, TMBPA and Methimazole in *S. tropicalis*

[a]Significantly greater ($p < 0.01$) than corresponding values for untreated tadpoles. [b]Significantly less ($p < 0.05$) than corresponding values for untreated tadpoles. [c]Significantly less ($p < 0.01$) than corresponding values for untreated tadpoles.
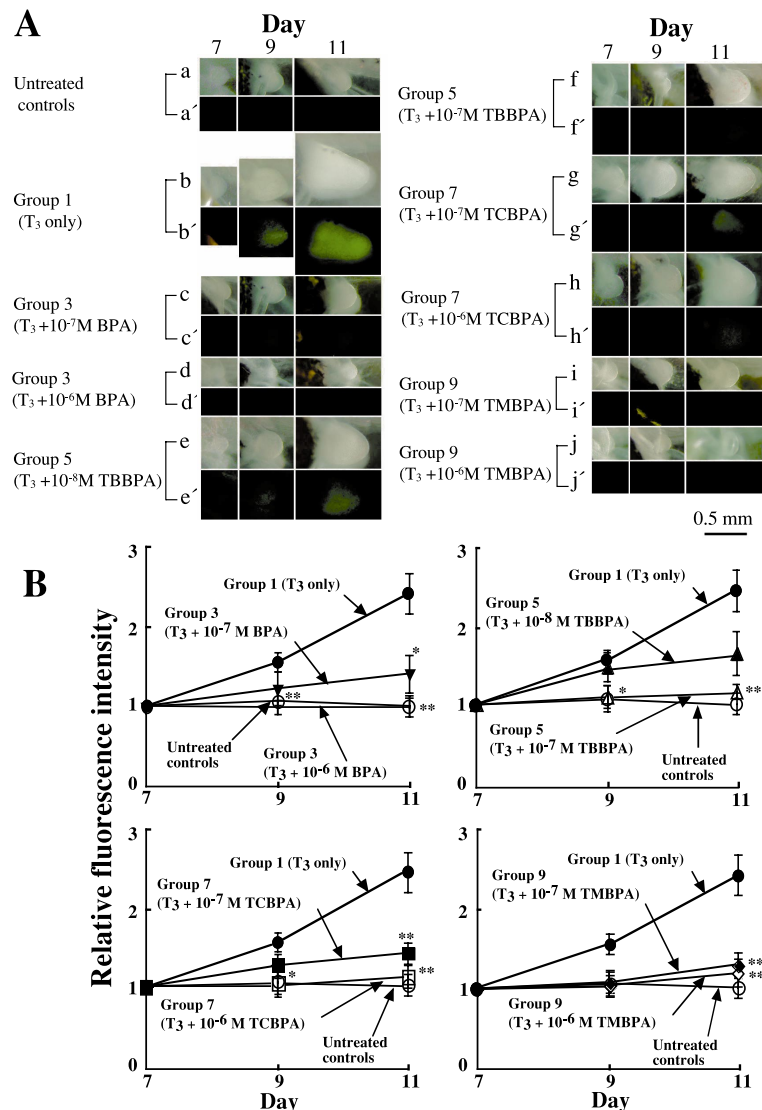
Fig. 5. EGFP Activity in Hindlimbs of T_3-Treated Transgenic *X. laevis* Tadpoles and Suppression by BPA, TBBPA, TCBPA and TMBPA
(A) Dorsal views of hindlimbs in reflected light (a–j) and EGFP fluorescence (a'–j'). (B) EGFP activity. *Significantly less ($p < 0.05$) than corresponding values for group-1 tadpoles. **Significantly less ($p < 0.01$) than corresponding values for group-1 tadpoles.

water served as untreated controls. The experiments were repeated three times. Figure 4 shows that BPA and related compounds suppressed spontaneous metamorphosis, tadpole tail shortening and hindlimb elongation, as did 1 mM methimazole.

Effects of BPA and Related Compounds on TR-Mediated Gene Transcription

The ability of BPA and related compounds to prevent TR-mediated gene expression was examined using F_2 transgenic *X. laevis* tadpoles expressing EGFP driven by TRβA1 promoter region containing TRE. EGFP fluorescence in the hindlimbs rose during treatment with T_3 (Fig. 5A), and activity increased about 250% over the original level by day 11

(Fig. 5B). The change in EGFP activity in T_3-treated tadpoles was significantly suppressed in the presence of BPA, TBBPA, TCBPA or TMBPA.

DISCUSSION

Although several classes of environmental contaminants (ECs) adversely disrupt thyroid function,[40,41] the reasons for this interference remain unclear. ECs have been found to disrupt thyroid gland function and regulation, TH metabolism, and TH transport binding proteins. A number of chemicals are thought to bind to the T_4-binding protein transthyretin (TTR), which displaces T_4, leading to

an increase in the clearance of T_4 and a decrease in serum T_4 concentrations.[42] TCBPA is reported to bind to TTR with lesser affinity than TBBPA.[43]

A few *in vitro* studies have been reported on the detrimental effects of BPA and related compounds on TH action. BPA inhibits T_3-induced differentiation of mouse oligodendrocytes.[44] In human embryonic kidney cells and hepatoblastoma cells, Moriyama *et al.*[5] found that BPA inhibits T_3 binding to TR and recruited nuclear receptor corepressors (N-CoRs) resulting in transcriptional suppression. TBBPA, TCBPA and TMBPA markedly inhibited T_3 binding to TR using nuclear fraction of GH3 cells.[11,12] Recently an *in vivo* investigation using rats showed that BPA acts as a TH antagonist on the *β*-TR and inhibits negative feedback, leading to elevated T_4 level.[45] Iwamuro *et al.*[6] reported that BPA suppresses spontaneous and T_3-induced metamorphosis in *X. laevis* tadpoles, blocking TR*β* gene expression.

In a recent investigation using reporter assay in a Chinese hamster ovary cell line (CHO-K1) transfected with TH*α*1 or *β*1, Kitamura *et al.*[12] reported that both TBBPA and TCBPA exhibit significant antithyroid hormone effects on T_3 activity. Kitamura *et al.*[12] also reported that TBBPA suppresses *R. rugosa* tadpole tails undergoing regression during T_3 enhancement. In the present study, BPA, TBBPA, TCBPA and TMBPA were found to inhibit the regression of *R. rugosa* tadpole tails induced by exogenously added T_3, as well as spontaneous *S. tropicalis* metamorphosis by endogenous circulating TH in a dose–dependent manner, suggesting that BPA and related compounds act as TH antagonists. TMBPA showed the highest activity, followed by TCBPA, TBBPA and BPA. Moreover, we found that exposure of T_3 to transgenic *Xenopus* tadpoles overexpressing TRE expression induced a marked expression of EGFP gene, while the addition of BPA, TBBPA, TCBBA or TMBPA blocked this EGFP expression in a dose–dependent manner. It seems likely that by binding to TR, these chemicals compete with T_3, resulting in suppression of TR-mediated gene transcription. Such transgenic lines overexpressing TH enhanced gene sets have proven to be a useful bioassay for detecting chemical compounds possessing TH antagonism or agonism.

Tadpole tail shortening through muscle cell apoptosis is one of the most spectacular events in anuran metamorphosis. We reported that an increase in thyroid hormone (T_4) enhances nitric oxide (NO) generation, thereby strongly inhibiting catalase activity, resulting in an increase in hydrogen peroxide (H_2O_2), and that the oxidative stress elicited by excess H_2O_2 might activate cysteine-dependent aspartate-directed protease-3 (caspase-3), which induces DNA fragmentation, leading to apoptosis.[25,26,46] We also reported that mitochondrial membrane permeability transition (MPT) is important in the mechanism of T_3-induced shortening of tadpole tails, and that tail muscle apoptosis is regulated by Bax gene in a cyclosporin A (CsA) sensitive mechanism.[27,47] At present, we are conducting investigations aimed at clarifying the effects of BPA and related compounds on NO generation and MPT in tail regression.

In the present study, we found that exposure of transgenic tadpoles to T_3 induced the expression of EGFP gene, while exposure of T_3 plus BPA, TBBPA, TCBPA or TMBPA suppressed EGFP expression, suggesting that BPA and related substances downregulate the gene expression mediated by TR. Transgenic lines exploiting TR-TRE are useful for investigating the effects of endocrine disrupting chemicals on TR-mediated gene transcription.

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