0013-7227/02/$15.00/0
*Printed in U.S.A.*

The Journal of Clinical Endocrinology & Metabolism 87(11):5185–5190

doi: 10.1210/jc.2002-020209

# Thyroid Hormone Action Is Disrupted by Bisphenol A as an Antagonist

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**Bisphenol A (BPA), a monomer of polycarbonate plastics, has been shown to possess estrogenic properties and act as an agonist for the estrogen receptors. Although an epidemiologically based investigation has suggested that some chemicals could disrupt thyroid function in animals, the effects on thyroid hormone receptors (TRs) are unknown. We show here that BPA inhibits TR-mediated transcription by acting as an antagonist. In the transient gene expression experiments, BPA suppressed transcriptional activity that is stimulated by thyroid hormone ($T_3$) in a dose-dependent manner. The inhibitory effects were observed in the presence of physiological concentrations of $T_3$. In contrast, in the case of negatively regulated TSH$\alpha$ promoter, BPA activated the gene transcription that is suppressed by $T_3$. To elucidate possible mechanisms of the antagonistic action of BPA, the effects on $T_3$ binding and cofactor interaction with TR were examined. The $K_i$ value for BPA was 200 $\mu$M when assessed by inhibition of [$^{125}$I]$T_3$ binding to rat hepatic nuclear TRs. In a mammalian two-hybrid assay, BPA recruited the nuclear corepressor to the TR. These results suggest that BPA could displace $T_3$ from the TR and recruit a transcriptional repressor, resulting in gene suppression. This is the first report that BPA can antagonize $T_3$ action at the transcriptional level. BPA may disrupt the function of various types of nuclear hormone receptors and their cofactors to disturb our internal hormonal environment. (*J Clin Endocrinol Metab* 87: 5185–5190, 2002)**

ENDOCRINE DISRUPTERS (ED), compounds that modify natural endocrine function, have emerged as a major public health issue. These effects are due to their potentially disruptive effects on physiological processes, particularly through direct interaction with steroid hormone receptors (1). In view of this situation it is important to determine whether a xenobiotic can mimic, block, or modify the effects of these hormones. One of targets of endocrine disrupters is thought to be nuclear hormone receptors, which bind to steroid hormones and regulate target gene transcription. Nuclear hormone receptors constitute a large superfamily of ligand-inducible transcriptional factors, which include receptors for steroid hormones, thyroid hormones, vitamin $D_3$, retinoids and prostanoids, and a number of proteins with high sequence homology but as yet unidentified ligands (2). Recent public and scientific interest has been mostly focused on environmental chemicals capable of interacting with the estrogen receptors (ERs). The effects of these compounds on the transcriptional activity of other nuclear hormone receptors have not been extensively studied. It is of great interest, therefore, to determine the effects of ED on these receptors to understand the mechanism of ED disruption of endocrine systems at the molecular level.

Bisphenol A (BPA) is a monomer of plastic materials that are widely used in daily life. BPA is detectable in our environment and is present in drinking water, canned goods, and even milk bottles. Recently, it was shown that BPA contaminates not only human plasma, but also fetal tissues (3). Many reports have shown that BPA has a weak effect to stimulate ERs. High doses of BPA may have reproductive toxicity and affect cellular development in rats and mice (4, 5). *In vitro*, BPA competes with estradiol for binding to ER$\alpha$ and introduces the expression of progesterone receptors and proliferation of MCF-7 human breast cancer cells (6–8). Thus, BPA has been shown to mimic estrogen both *in vivo* and *in vitro* as a xenoestrogen. In contrast, polychlorinated biphenyls (PCBs), a class of industrial compounds, are environmentally persistent and bioaccumulative agents that have been shown to affect a number of endocrine targets (9). PCB-induced disruption of thyroid function is thought to be due to their toxicological effects, which may be related to the structural similarities shared by PCBs and thyroid hormone (10, 11). The influences of BPA on the thyroid system are unknown.

The functions of $T_3$ are mediated by several isoforms of nuclear TRs, TR$\alpha$1, and TR$\beta$1–3 encoded by two genes, *TR$\alpha$* and *TR$\beta$*, respectively (12–14). The *TR$\alpha$* locus generates TR$\alpha$1 and several related proteins, TR$\alpha$2, TR$\alpha$3, and TR$\Delta\alpha$s, which result from alternative splicing of the TR$\alpha$ primary transcript (15, 16). The *TR$\beta$* locus generates TR$\beta$1, TR$\beta$2, TR$\beta$3, and TR$\Delta\beta$s by using different promoters and alternative splicing. TR$\beta$1, TR$\beta$2, and TR$\beta$3 have an identical ligand binding domain (LBD). The expression of TR$\beta$2 is restricted to some specific organs, including the pituitary and hypothalamus, where it appears to play a key role in the regulation of TSH

Abbreviations: BPA, Bisphenol A; CoR, corepressor protein; DBD, DNA binding domain; ED, endocrine disrupters; ER, estrogen receptor; h, human; LBD, ligand binding domain; Luc, luciferase; ME, malic enzyme; N-CoR, nuclear receptor corepressor; PCB, polychlorinated biphenyl; tk, thymidine kinase; TR, thyroid hormone receptor; TRE, thyroid hormone response element; UAS, upstream activation site.

synthesis and secretion. In contrast, the tissue distributions of TR$\alpha$1, TR$\beta$1, and TR$\beta$3 are relatively ubiquitous (14, 17, 18), and the expression of these proteins begins early in development (19–23).

Here we report that BPA can disturb thyroid hormone action. BPA reduced $T_3$ binding to the nuclear TRs and recruited nuclear receptor corepressors (N-CoRs) to the TR, resulting in transcriptional inhibition.

## Materials and Methods

### *$T_3$ binding studies*

Nuclear TRs were prepared from the Sprague Dawley rat liver as previously described (24). A tracer dose of [$^{125}$I]$T_3$ (122 MBq/$\mu$g; NEX-110X, NEN Life Science Products, Boston, MA) and nuclear TRs in 5 mM dithiothreitol were incubated with BPA (Sigma, St. Louis, MO) at 4 C overnight. Bound and free [$^{125}$I]$T_3$ were separated by adding 1 ml 2% Dowex resin (Supelco, Bellefonte, PA) suspension. The nonspecific binding obtained in the presence of an excess of $T_3$ was subtracted from the total binding.

### *Plasmid constructions*

Expression vectors containing wild-type human TR$\beta$1 [pCMX-human (h) TR$\beta$1] and human TR$\alpha$1 (pCMX-hTR$\alpha$1) were provided by K. Umesono (The Salk Institute, San Diego, CA) (25). The plasmid pCMX-rTR$\beta$2 contains rat TR$\beta$2 cDNA (26). The LBD of hTR$\alpha$1 or hTR$\beta$ was fused to the DNA binding domain (DBD) of Gal4 in-frame in pSG424 (27). The Gal4-NCoR (residues 1552–2453) construct contains the TR interaction domains of N-CoR (28). The VP16 construct for hTR$\beta$ contains the LBD of the receptor downstream of the VP16 activation domain of herpes simplex virus in-frame in pCMX (29). The plasmids, thyroid hormone response element (TRE)-thymidine kinase (tk)-luciferase (Luc) (30) and malic enzyme (ME)-TK-Luc (31) contain two copies of a palindromic TRE and the ME-TRE, respectively, upstream of the tk promoter (tk109) in the pA3 luciferase vector (30), and the Gal4 reporter plasmid, upstream activation site (UAS)-E1BTATA-Luc, contains five copies of the UAS element upstream of E1BTATA in pA3-Luc (28). The pRL-tk vector (Promega Corp., Madison, WI) comprised of the tk promoter and *Renilla* luciferase cDNA was used as an internal control.

### *Transient expression assays*

TSA 201 cells, a clone of human embryonic kidney 293 cells (32), and human hepatoblastoma cells (HepG2) were grown in phenol red-free DMEM (Nikken, Kyoto, Japan) with 10% charcoal-stripped fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml) and were transfected using the calcium precipitation method (26) for TSA201 or lipofectin (Lipofectamine Plus, Invitrogen, Carlsbad, CA) for HepG2, according to the manufacturer's instructions. After exposure to the DNA precipitate for 8 h, phenol red-free DMEM with charcoal-stripped FBS was added in the absence or presence of BPA and/or $T_3$. Cells were harvested for measurements of Luc activity according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega Corp.). The transfection efficiencies were corrected with the internal control. Results are expressed as the mean ± SE from at least three transfections, each performed in triplicate. Data were analyzed by *t* test to compare with the control.

## Results

### *BPA is a weak ligand for TR*

The chemical structures of BPA and $T_3$ are shown in Fig. 1, A and B, respectively. There is an unexpected resemblance between them. Two benzene cores are linked by carbon (BPA) or oxygen ($T_3$). BPA has two hydroxyl groups, and $T_3$ has a hydroxyl and an alanine group. BPA displaced [$^{125}$I]$T_3$ from endogenous TR, which is prepared from the rat liver, with an inhibition constant ($K_i$) of 200 $\mu$M (Fig. 1C). Scatchard

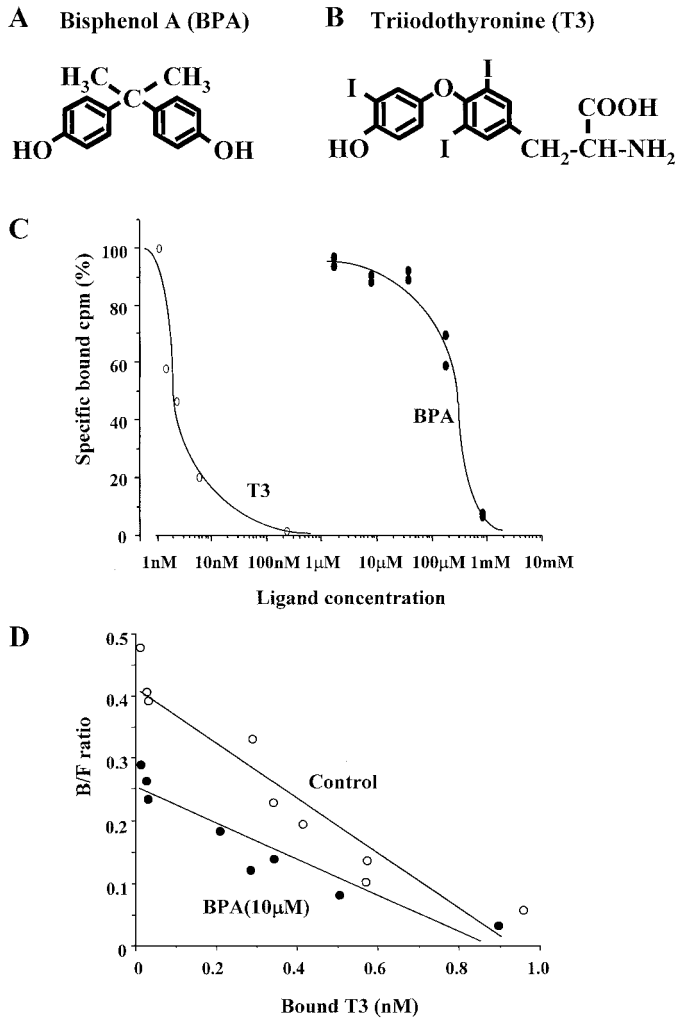


FIG. 1. Binding of BPA to nuclear TR. A and B, Comparison of structures of BPA (A) and $T_3$ (B). C, Competition binding assay. Rat liver nuclear extract (50 $\mu$g protein) was incubated with a tracer dose of [$^{125}$I]$T_3$ and increasing amounts of $T_3$ or BPA. D, Scatchard analysis. Rat liver nuclear extract (50 $\mu$g protein) was incubated with various amounts of [$^{125}$I]$T_3$ in the presence or absence of 10 $\mu$M BPA. B, Bound; F, free.

analysis revealed that BPA decreased the value for the association constant ($K_a$) from 0.44 to 0.28 $\times$ $10^9$ M, whereas little effect was observed on maximum binding capacity (Fig. 1D).

### *BPA suppressed transcriptional activities mediated by TR$\alpha$1 and TR$\beta$1*

Transient expression experiments were performed using TSA201 cells, which are a derivative of human embryonic kidney 293 cells. The LBD of TR$\alpha$1 or TR$\beta$ was fused to the DBD of the yeast transcription factor, Gal4, and was cotransfected with a Gal4 reporter gene, UAS-E1BTATA-Luc. Although BPA may bind to the TR, BPA did not activate TRs (data not shown). We examined whether BPA antagonizes $T_3$-induced TR activation. In the presence of 3 nM $T_3$, dose-dependent inhibition of transcription mediated by Gal4-TR$\alpha$1 (Fig. 2A) and Gal4-TR$\beta$ (Fig. 2B) was observed. BPA

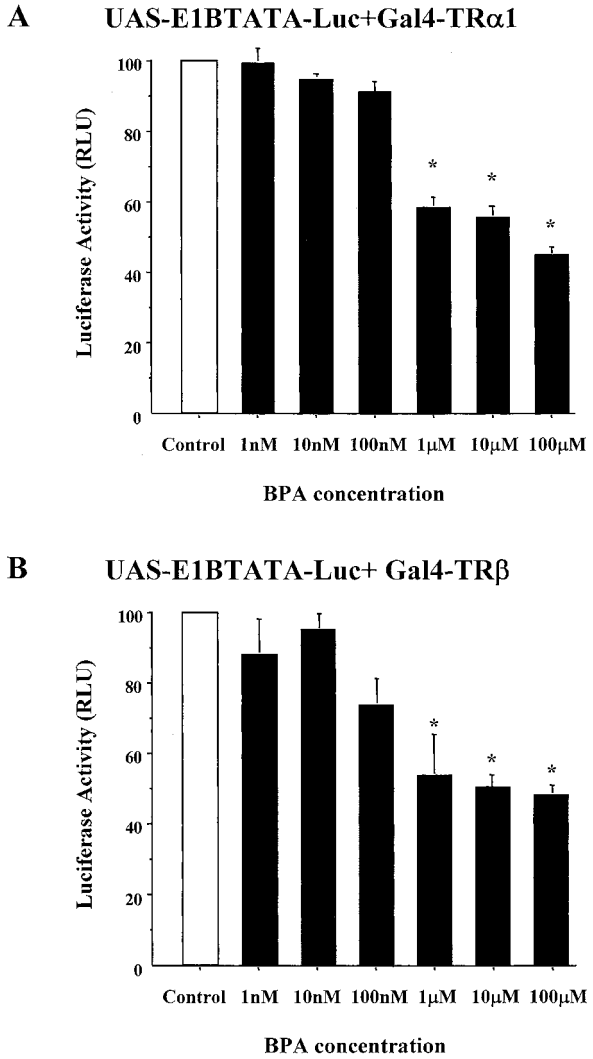


FIG. 2. The inhibitory effects of BPA on the gene transcription mediated by the TR-LBD. Gal4-TR$\alpha$1 (A) or Gal4-TR$\beta$ (B; 50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the presence of 3 nM $T_3$ and increasing amounts of BPA. RLU, Relative light units. *, $P < 0.01$ *vs.* control.

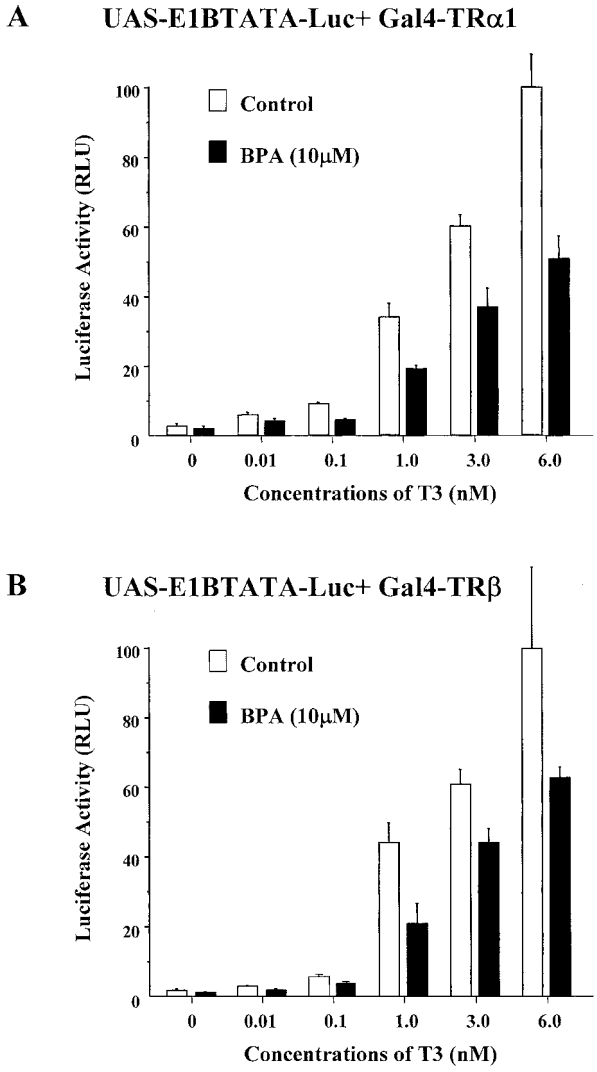


FIG. 3. BPA suppresses TR-mediated transcription in the presence of a physiological range of $T_3$. Gal4-TR$\alpha$1 (A) or Gal4-TR$\beta$ (B; 50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the absence or presence of 10 $\mu$M BPA. RLU, Relative light units.

had no significant effect on the basal transcriptional activity mediated by Gal4-DBD alone (data not shown).

We next determined the effects of BPA on various physiological concentrations of $T_3$. In the presence of 10 $\mu$M BPA, increasing amounts of $T_3$ were added to the medium, and transcriptional activity was measured. As shown in Fig. 3A, BPA suppressed the activity mediated by Gal4-TR$\alpha$1 up to about 50% of the respective control level. Similar results were obtained using Gal4-TR$\beta$ (Fig. 3B).

The inhibitory effects of BPA were also examined in the context of native receptors. A $T_3$-responsive reporter gene, TRE-tk-Luc, was cotransfected with full-length TRs. Increasing concentrations of BPA significantly suppressed the transcriptional activities mediated by TR$\alpha$1 (Fig. 4A) and TR$\beta$1 (Fig. 4B). In a reciprocal manner, another group of negatively regulated genes was stimulated by TRs in the absence of $T_3$ and was repressed in response to $T_3$ (26). The effects of BPA on the TSH$\alpha$ promoter were examined as a model of a negatively regulated gene. As shown in Fig. 4C, BPA increased the transcriptional activity, which was already suppressed by 10 nM $T_3$. The stimulating effects were observed in the presence of TR$\beta$1 as well as TR$\beta$2, which is expressed mainly in the pituitary and hypothalamus.

### *BPA suppressed transcriptional activities mediated by endogenous TRs*

We next studied the effects of BPA using a cell line that contains physiological amounts of endogenous TRs. The reporter gene regulated by the ME-TRE, ME-tk-Luc, was transfected into human hepatoblastoma cells, HepG2. Twenty-four-hour incubation with 10 nM $T_3$ stimulated the expression of ME-tk-Luc by 1.7-fold (Fig. 5). Addition of 10 $\mu$M BPA significantly decreased gene transcription to 78.7% of that with 10 nM $T_3$ alone.

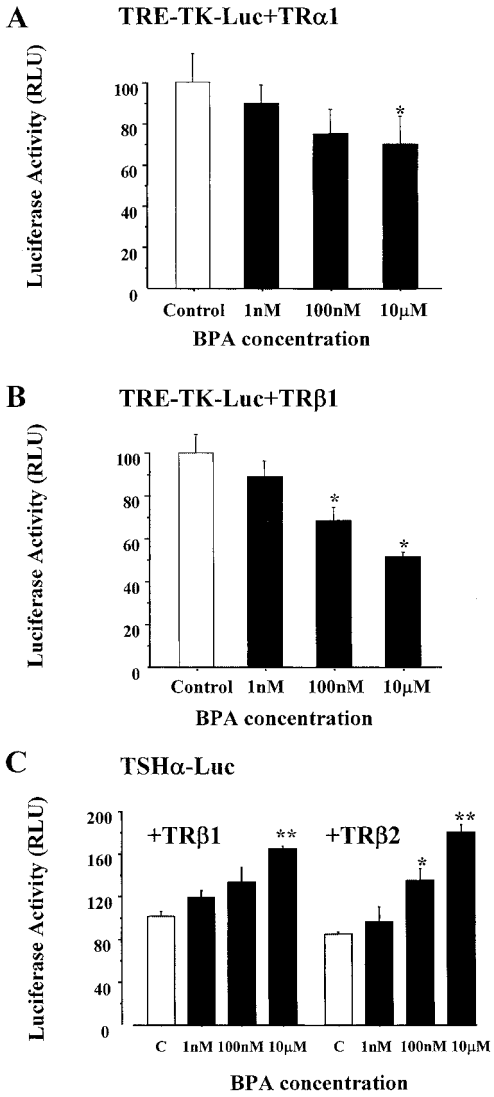


FIG. 4. The inhibitory effects of BPA on the gene transcription mediated by the native TRs. A and B, CMX-TR$\alpha$1 (A) or CMX-TR$\beta$1 (B; 10 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TRE-tk-Luc, in the presence of 10 nM $T_3$ and increasing amounts of BPA. C, CMX-TR$\beta$1 or CMX-TR$\beta$2 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TSH$\alpha$-Luc, in the presence of 10 nM $T_3$ and increasing amounts of BPA. RLU, Relative light units. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control).

*BPA recruits N-CoR*

Transcriptional repression of the positively regulated genes by unliganded TR is mediated by interacting with corepressor proteins (CoRs). CoRs might also be involved in the basal activation of negatively regulated genes (26). Using a mammalian two-hybrid assay, the effect of BPA on the TR-CoR interaction was examined. The carboxy-terminal half of a CoR, N-CoR, which contains TR interaction domains, was fused to the Gal4-DBD. The LBD of TR$\beta$1 was fused to the transcriptional activation domain of VP16 to allow detection of the interaction between the Gal4-NCoR and VP16-TR. Although increasing concentrations of $T_3$ decreased the interaction between these proteins

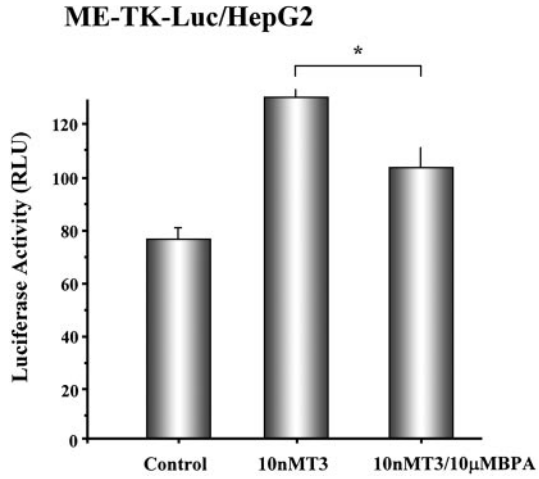


FIG. 5. BPA suppressed transcriptional activities mediated by endogenous TRs. The ME-tk-Luc (100 ng) was transfected into HepG2 cells and incubated with or without 10 nM $T_3$ and/or 10 $\mu$M BPA for 24 h. RLU, Relative light units. *, $P < 0.05$.

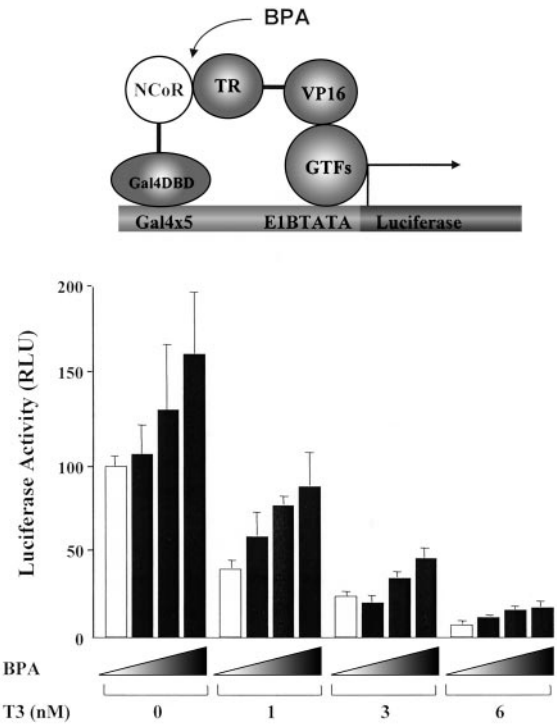


FIG. 6. The effect of BPA on the interaction between TR and N-CoR. The format of the mammalian two-hybrid experiment is shown at the *top* of the figure. Gal4-NCoR (50 ng) were cotransfected into TSA-201 cells with 100 ng VP16-TR together with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the absence or presence of $T_3$. Increasing amounts of BPA (1 nM, 100 nM, and 10 $\mu$M) were added. RLU, Relative light units.

(indicated by □ in Fig. 6), BPA enhanced those interactions in a dose-dependent manner (■).

## Discussion

BPA is detected in human plasma, cord sera, and even fetal tissues (3, 33). The concentration was more than 1 ng/g wet

weight of the umbilical cord. Serum BPA concentrations were reported to be 1.49 ± 0.11 ng/ml in men and 0.64 ± 0.10 ng/ml in women (34). The Japanese Ministry of Health and Welfare has established the standards for regulations against BPA levels in food containers. The upper limit of emission is set to 2.5 ppm ($\mu$g/liter), which is more than 90 $\mu$M. This level corresponds to world standards. The results in this study indicate that concentrations even below the upper limit can interfere with thyroid hormone action *in vitro*.

Thyroid hormones are essential for normal behavioral, intellectual, and neurological development. Congenital hypothyroidism if left untreated causes irreversible mental retardation. Even mild maternal thyroid deficiency during pregnancy could cause retarded neurological development of the child (35). There is increasing evidence that exposure to certain synthetic compounds, such as dioxins and PCBs, during the perinatal period can impair normal thyroid function. PCBs reduced circulating and tissue thyroid hormone concentrations using animal experiments (36, 37), and dioxins and PCBs were observed to alter thyroid hormone status by epidemiological investigations (38). The PCB-induced reduction in circulating $T_4$ has been attributed to increased excretion of free $T_4$ due to competitive binding of PCBs with thyroid hormone transport proteins (10, 39), amplified biliary excretion of $T_4$ by induction of UDP-glucuronosyltransferase (40), and/or direct damage to the thyroid gland (41). Thus, a given ED can interfere with thyroid hormone functions and homeostasis by inhibiting hormone synthesis, altering serum transport proteins, or increasing catabolism of thyroid hormones. Regarding gene transcription, there are no direct data to support the assertion that certain ED may alter thyroid hormone action.

The effects of BPA on thyroid function have not been elucidated. In contrast, the estrogenicity of BPA has been demonstrated in a number of *in vitro* and *in vivo* assays. *In vitro* assay end points include binding to the ER (42, 43) and activation of ERE-driven reporter gene constructs (44). Upon iv injection of BPA into rats, levels of BPA were determined in serum and various organs (45). BPA was detected predominantly in the lung, followed by kidneys, thyroid, stomach, heart, spleen, testes, liver, and brain. Ratios of the organ to serum BPA concentrations exceeded unity for all organs examined (ratio range, 2.0–5.8), except for brain (ratio, 0.75). Thus, BPA has the potential to interfere with thyroid hormone action in each organ accumulated by BPA. The *in vivo* effects of BPA are under investigation using experimental animals.

In this study we demonstrated that BPA could impair thyroid hormone action by inhibiting $T_3$ binding to the TR and by suppressing its transcriptional activity. Gene suppression is attributed partly to the recruitment of N-CoR to the TR by BPA. In contrast, some compounds exert their estrogen-like activity through the ER by recruiting coactivators, such as SRC1 and RIP140, in a manner similar to that of estradiol (46, 47). A number of nuclear cofactors have been cloned, but most of their specific functions are unclear (48). Moreover, more than 150 nuclear receptors may exist in mammalian cells as targets for ED. Indeed, BPA activated the transcription mediated by the human orphan receptor, steroid and xenobiotic receptor (49), but not by its mouse ortholog, pregnane X receptor (50). BPA also did not activate the transcription mediated by androgen, progesterone, glucocorticoid, or mineralocorticoid receptors (46). Increasing concerns over the effects of environmental hormones highlight the need for screening of the effects of ED on nuclear receptors to assess potential disruption of the endocrine system.

In summary, our findings demonstrate that BPA, which is one of the most prevalent chemicals for daily use materials, suppresses transcriptional activity by inhibiting $T_3$ binding to the TR and by recruiting N-CoR on the promoter. Further studies, such as animal experiments and epidemiological investigations, will allow evaluation of the effects of BPA on the human endocrine system.

## Acknowledgments

We thank Ms. Keiko Matsuda, Ms. Maki Kouchi, and Ms. Hitomi Hiratani for their excellent secretarial assistance. We are also grateful to Dr. J. Larry Jameson for comments and suggestions on the manuscript.

Received February 12, 2002. Accepted August 14, 2002.

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This work was supported by grants from Asahi Brewers Foundation and the Foundation for Total Health Promotion.

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