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Dibutyl Phthalate Contributes to the Thyroid Receptor Antagonistic Activity in Drinking Water Processes

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It has long been recognized that thyroid hormone (TH) is essential for normal brain development in both humans and animals, and there is growing evidence that environmental chemicals can disrupt the thyroid system. In the present work, we used a two-hybrid yeast assay to screen for agonistic or antagonistic thyroid receptor (TR) mediated effects in drinking waters. We found no TR agonistic, but TR antagonistic activities in all samples from the drinking water processes. The TR antagonistic activities in organic extracts of water samples were then calibrated regarding to a known TR-inhibitor, NH3, and were expressed as the NH3 equivalents (TEQbio). The observed TEQbio in waters ranged from 180.8 ± 24.8 to 280.2 ± 48.2 μg/L NH3. To identify the specific compounds responsible for TR disrupting activities, the concentrations of potentially thyroid-disrupting chemicals including organochlorine pesticides (OCPs), phenols, and phthalates in organic extracts were quantitatively determined and their toxic equivalents with respect to NH3 (TEQcal) were estimated from their concentration-dependent relationships, respectively, using the same set of bioassays. Based on the TEQ approach, it was revealed that dibutyl phthalate (DBP) accounted for 53.7 ± 8.2% to 105.5 ± 16.7% of TEQbio. There was no effective removal of these potential thyroid disrupting substances throughout drinking water treatment processes.

Introduction

In recent years, an increasing number of environmental contaminants have been found to disrupt the endocrine system in wildlife and humans (*1*). Most research has focused on estrogenic effect and far less attention has been paid to identifying compounds with thyroid disrupting activity in environment. However, in recent years, there has been an increasing concern on the effects of synthetic chemicals on the thyroid system, because of abundant evidence both from in vivo and in vitro studies demonstrating that the thyroid system is also vulnerable to endocrine-disrupting effects (*2*).

Thyroid hormones (THs) regulate growth, energy metabolism, tissue differentiation, reproduction, and formation of the central nervous system (*3*). Normal thyroid hormone levels are essential for humans and the changes of normal hormone levels can adversely affect pregnancy outcome, fertility, and postnatal development in humans and animals (*4*). Especially when changes occur in a critical developmental phase, the neuropsychological development of a child can be adversely affected, and may cause profound and irreversible damage to the newborn (*5*).

Increasing evidence from in vivo and in vitro studies demonstrate that many environmental pollutants have been reported to affect the thyroid hormone system (*6*−*8*). This is particularly important considering that many of the thyroid hormone system disrupting chemicals can directly interfere with thyroid receptors (TRs), either by decreasing normal ligand (T_3) binding or by providing additional ligands that may bind to TRs (*9*). In our previous work, Li et al. (*10*) developed a novel screening method for chemicals with TR ant/agonistic properties using a yeast two-hybrid system, and found a lot of chemicals such as polychlorinated biphenyls (PCBs), flame retardants, phthalates, pesticides, and phenols have the agonistic or antagonistic activities with TR. Given that the major mechanism of TH action involves T_3 binding to TRs, resulting in tissue-specific activation/repression of gene transcription (*11*), assessing the environmental pollution interfering with TRs is of great importance.

Recently, EDCs are emerging as being of major concern for water quality, as multiple EDCs have been detected in wastewater effluents and surface waters (*12*). EDCs can even enter drinking water sources and can be detected in human bodies (*13, 14*), and endocrine system of human may be affected (*15*). As it is frequently not possible to completely remove the contaminants by water treatments, natural waters often contain many hormone disrupting chemicals impacting drinking water supplies (*16*). Thus there is a growing concern over the fate of hormone disrupting chemicals during drinking water treatment. Of these TR disrupting chemicals mentioned above, some organochlorine pesticides (OCPs) and phenols were detected in the drinking water in Beijing in our previous work (*17*), and PAEs were reported to have caused serious pollution in Beijing (*18*). Diethylhexyl phthalate (DEHP) was detected in the groundwater in Beijing up to 241.8 μg/L (*19*). Therefore, the aim of this study was to assess the TR mediated effects in drinking water processes and try to identify the specific compounds responsible for TR disrupting activities.

Materials and Methods

Chemicals. NH3 (>95%) was offered by Dr. Thomas S. Scanlan. 3,3′,5-triiodo-L-thyronine (T_3, 95%), desethyl amiodarone hydrochloride (DAH, 98%), dimethyl sulfoxide (DMSO, 99.5%), dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), diethy phthalate (DEP), benzyl butyl phthalate (BBP), pentachlorophenol (PCP, 99%), *r*-hexachlorocyclohexane (*r*-HCH, 99%), methoxychlor (*o,p′*-DDT, 99%), 1-chlor-2-(2,2,2-trichlor-1-(4-chlorphenyl)ethyl)benzol (*p,p′*-DDT, 99%), *p,p′*-dichlorodiphenyldichloroethane (*p,p′*-DDD, 99%), *p,p′*-dichlorodiphenylethane (*p,p′*-DDE, 99.6%) were purchased from Sigma Chemical (St. Louis, MO). Amiodarone hydrochloride (95%) was bought from Shanghai Pharmaceutical (Shanghai, China). HPLC grade dichloromethane, hexane, methanol, and tert-butyl methyl ether were purchased from Fisher Scientific (Fair Lawn, NJ). 4-Aminophenol (4-AP, 97.5%) and 2,4-dichlorophenol (2,4-DCP, 99%) were purchased from Acros Organics (Belgium).

Sample Collection and Processing. Composite sampling (24 h) was conducted in May 2007 at a waterworks with a total capacity of 1,500,000 m^3/d located in Beijing, China. Samples were collected from a reservoir that supplied the source water located in Beijing and the effluents from the treatment processes, including prechlorination, coagulation,

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coal and sand filtration, activated carbon filtration, and secondary chlorination.

Each of the water samples (20 L, 10 L for bioassay and 10 L for chemical analysis) of treatment processes mentioned above and the source water were collected in precleaned amber glass bottles. Prior to use the bottles were soaked overnight by 10% nitric acid and soaked by chromic acid solution for 30 min, then washed three times by double-distilled water and methanol. The bottles were also washed 3 times with treatment process samples before sample collection. An appropriate amount of methanol (2 mL/L) was added in each sample right after sampling to suppress possible biotic activities. Samples were stored at 4 °C prior to treatment. All samples were treated within 48 h.

Water samples and procedure blanks (Mili-Q water, conductivity of 18.2 Ω) were filtered with glass fiber filters (0.45-μm, Whatman, England) to remove insoluble materials. Then solid phase extraction (SPE) was performed using 500-mg Oasis HLB cartridges (Waters, USA) conditioned according to the manufacturer's directions. The cartridges were forced under vacuum at a flow rate of approximately 6 mL/min. The cartridges were then kept under vacuum aspiration for 5 min to dry any residual water. The cartridges for bioassay were washed with 5 mL of hexane/dichloromethane (7/3) twice, 5 mL of tert-butyl methyl ether twice, 5 mL of dichloromethane/methanol (9/1) twice, and 5 mL of methanol at a flow rate of 1 mL/min; the extracts were then combined. The cartridges for chemical analysis were washed with 10 mL of dichloromethane/methanol (9/1). Then the extracts were filtered by anhydrous sodium sulfate to remove water and evaporated to dryness in a rotary evaporator (R-200, Buchi, France) at 40 °C to 2 mL. Then the dehydrated extracts were blown to dryness under gentle nitrogen flow and reconstituted in 0.1 mL of DMSO for bioassay immediately and 0.5 mL of hexane for chemical analysis. Finally extracts were stored at −20 °C in glass vials. Details of chemical analysis and quality control are described in the Supporting Information (S1−S3).

Bioassay. The bioassays including agonistic activity test and antagonistic activity test were conducted using yeast strain hTR-GRIP1 as described by Li et al. (*10*). All experiments were carried out in triplicate. Each assay group included the sample, the positive control (T_3 for agonistic activity or T_3 + NH3 for antagonistic activity), the negative control (DMSO), and the procedural blank. Serial dilutions (5 μL) of test samples were combined with 995 μL of medium containing 5×10^3 yeast cells/mL resulting in a test culture in which the volume of DMSO did not exceed 1.0% of the total volume. For determination of agonistic activities, the extracts were tested in the absence of T_3; and for antagonistic activities they were tested in presence of 5×10^{-7} mol/L of T_3 which produced a submaximal stimulatory response (*20*). The β-galactosidase activity was calculated according to equations described by Gaido et al. (*21*).

The control assays of the yeast assay were performed for the procedure blank. It can be seen in Figure 1 that all of the induction or inhibition activities of procedure blanks were less than 5% and no concentration-dependent relationships were found. To exclude the false results caused by cytotoxicity, the cell viability was determined spectrophotometrically as a change of cell density (OD_{600} nm) in the assay medium. The procedural blank was tested in the same concentration to monitor for a false-positive result. The detailed steps have described before (*10*).

Causality Analysis. To identify the specific compounds responsible for TR disrupting activities, potentially thyroid-disrupting chemicals in the waters were quantified and causality analysis was performed on the TEQ approach (*22*). Shortly, the concentration-dependent curve on inhibition of β-galactosidase expression by NH3 in presence of submaximal T_3 concentration was obtained. Then the responses from extracts were expressed according to the concentration-dependent curve of NH3, which was then converted to its water concentration and obtained the bioassay derived equivalent concentrations (TEQbio). The extract was diluted to fit the linear part of the concentration-dependent curve for NH3. Similarly, the concentration-dependent curves were constructed for detected OCPs, phenols, and phthalates, and their toxic equivalents with respect to NH3 were calculated from their concentrations in the water samples (TEQ_{cal}). Relative potencies (REPs) of detected chemicals were calculated. The contribution of each of the potential TR antagonists to the TEQ_{bio} was then estimated.

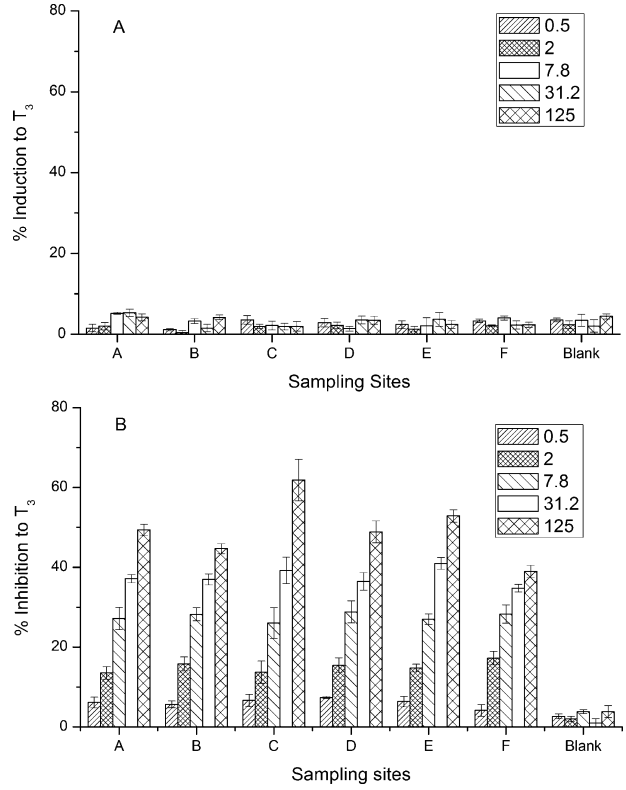

FIGURE 1. Concentration-dependent relationships of thyroid receptor (TR) disrupting effects of water extracts. The agonistic activities (A) and antagonistic activities (B) of water extracts were determined by the TR yeast bioassay and represented as the percent induction and inhibition activity relative to the maximum induced by 3,3′,5-triiodo-L-thyronine (T_3, 5×10^{-7} mol/L). Values are presented as the average ± standard error (n = 3). A: source water, B: effluent of prechlorination, C: effluent of coagulation, D: effluent of coal and sand filtration, E: effluent of activated carbon, F: finished water after secondary chlorination. 0.5, 2, 7.8, 31.2, and 125 mean the concentration folds of the original water.

Results and Discussion

To choose a proper chemical as positive control, we tested the NH3 and desethylamiodarone hydrochloride besides amiodarone hydrochloride which had been tested in our previous work (*10*). In the present study, NH3 and desethylamiodarone hydrochloride (Figure 2A) both inhibited the β-galactosidase activity induced by T_3 in concentration-dependent manners. NH3 and desethylamiodarone hydrochloride both inhibited less than 40% of induced activity by T_3 (5×10^{-7} mol/L) at concentration levels of 1×10^{-5} mol/L which are similar to amiodarone hydrochloride. NH3 was reported to apparently interact with the TR ligand binding domain and inhibit coactivator recruitment (*23*). Desethy-

FIGURE 2. Concentration-dependent relationships of DBP, DEHP, DEP, NH3, and DAH (A) and bioassay-derived NH3 equivalence of the samples' antagonistic activities (NH3-EQbio) (B). The chemicals determined by the yeast strain human thyroid hormone receptor (hTR)-GRIP1 for thyroid hormone receptor (TR) antagonistic activity. The chemicals' antagonistic activities are represented as the percent inhibition activities relative to the maximum induced by 3,3′,5-triiodo-L-thyronine (T_3). Values are presented as the average ± standard error (n = 3). $NH3\text{-}EQ_{DBP}$ denotes the toxic equivalents of DBP. DBP: dibutyl phthalate, DEHP: diethylhexyl phthalate, DEP: diethy phthalate, DAH: desethyl amiodarone hydrochloride. Footnotes of A, B, C, D, E, and F are as the same as in Figure 1.

lamiodarone hydrochloride which is the metabolite of amiodarone hydrochloride has been shown to be a non-competitive inhibitor of TRs (*24*). Thus we chose NH3 as the positive control in the present study.

In the present study, no TR agonistic activities were observed even when the water samples were 125 times concentrated (Figure 1A). However, all samples had TR antagonistic activities in concentration-dependent manners even when the water samples were 7.8 times concentrated (Figure 1B.). The sample of procedure blank had no TR disrupting activity as shown in Figure 1A and B. The TEQbio of water sample was calculated according to the method in the experimental section and ranged from 180.8 ± 24.8 to 280.2 ± 48.2 μg/L NH3. In the Supporting Information (Figures S4−S6), it is shown that the drinking water extracts have no general suppressive effects on TR gene expression and the inhibition activities of the drinking water extracts were specific to TR.

To identify causality for TR antagonists, the concentrations of OCPs, phenols, and phthalates in water extracts were determined and their TEQcals were estimated. Concentrations of OCPs and phenols in the water samples were below 6.0 ng/L (Table 1) and the REPs of OCPs and phenols detected in the drinking water were all below 1.0. Therefore, these two

TABLE 1. Concentrations of Chemicals in Drinking Water, Beijing, China[a]

concentrations at sampling sites	phenols (ng/L)				pesticides (ng/L)				phthalates (μg/L)			
	4-AP	2,4-DCP	PCP	γ-HCH	*p,p′*-DDE	*p,p′*-DDT	*p,p′*-DDD	*o,p′*-DDT	DBP	DEHP	DEP	BBP
A (source water)	1.2 ± 0.1	3.6 ± 0.3	5.3 ± 0.5	0.6 ± 0.02	N.D.	N.D.	N.D.	N.D.	15.6 ± 0.8	7.1 ± 0.3	1.0 ± 0.1	N.D.
B (prechlorination)	N.D.	3.4 ± 03	5.3 ± 0.5	0.7 ± 0.03	N.D.	N.D.	N.D.	N.D.	35.6 ± 0.2	9.6 ± 0.5	1.2 ± 0.1	N.D.
C (coagulation)	4.5 ± 0.3	4.3 ± 0.3	5.3 ± 0.5	0.6 ± 0.02	N.D	N.D.	N.D.	N.D.	17.5 ± 0.9	8.7 ± 0.4	1.3 ± 0.1	N.D.
D (coal and sand filtration)	N.D.	3.4 ± 0.3	5.3 ± 0.5	0.5 ± 0.02	N.D.	N.D.	N.D.	N.D.	28.7 ± 0.2	14.4 ± 0.7	1.0 ± 0.1	N.D
E (activated carbon)	N.D.	3.4 ± 0.3	5.3 ± 0.5	0.5 ± 0.02	N.D	N.D.	N.D.	N.D.	37.0 ± 2.0	6.9 ± 0.3	0.8 ± 0.1	N.D.
F (secondary chlorination)	N.D.	N.D.	5.3 ± 0.5	0.6 ± 0.02	N.D.	N.D.	N.D.	N.D.	28.9 ± 1.5	8.5 ± 0.4	1.3 ± 0.1	N.D.
blank	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
RIC20 (mol/L)	1.0×10^{-4} [b]	2.0×10^{-5} [b]	1.10×10^{-6} [b]	2.00×10^{-7} [b]	-	-	N.C.	N.C.	3.80×10^{-8}	2.40×10^{-6}	-	N.C.
REP	0.001	0.006	0.10	0.56	-	-	N.C.	N.C.	3.68	0.09	-	N.C.
LODs	0.1	0.5	0.2	0.3	0.7	0.4	1.3	0.6	0.2×10^{-3}	0.1×10^{-3}	0.3×10^{-3}	0.6×10^{-3}
LOQs	0.1	1.5	0.6	0.9	2.1	1.2	3.9	1.8	0.6×10^{-3}	0.3×10^{-3}	0.9×10^{-3}	1.8×10^{-3}
recovery (%)	86.2	84.7	91.7	57.7	100.2	105.5	105.3	83.5	105.0	106.0	80.6	80.5
RSD (%)	6.1	8.0	9.4	4.3	10.3	9.2	11.2	6.1	5.3	4.7	5.8	2.8

[a] 4-AP: 4-aminophenol; DCP: dichlorophenol; PCP: pentachlorophenol; HCH: hexachlorocyclohexane; *p,p′*-DDE: *p,p′*-dichlorodiphenylethane; *p,p′*-DDT: *p,p′*-dichlorodiphenyltrichloroethane; *p,p′*-DDD: *p,p′*-dichlorodiphenyldichloroethane; *o,p′*-DDT: methoxychlo; DBP: dibutyl phthalate; DEHP: diethylhexyl phthalate; DEP: diethy phthalate; BBP: benzyl butyl phthalate; RIC20: 20% relative inhibitory concentration; N.D.: not detected; N.C.: no consideration; "-": no effect; REP: relative potencies = RIC20 NH3/RIC20 compound *x*; LODs: Limits of detection (S/N = 3); LOQs: Limits of quantitation (S/N = 9); RSD: relative standard deviation. [b] According to Li et al. (*10*).

types of chemicals may not account for the observed antagonistic activities. Even the mixture toxicity should be emphasized.

Three phthalates could be detected in the water samples, i.e., DEP, DBP, and DEHP. As shown in Figure 2A, DEP showed no TR antagonistic activities and both DBP and DEHP demonstrated TR antagonistic activities in concentration-dependent manners from 1×10^{-9} mol/L to 1×10^{-4} mol/L. The TR antagonistic activities of DBP and DEHP in the present study were quite similar to those reported by Shen et al. (*25*)

The concentration of DBP and DEHP in water samples ranged from 6.9 ± 0.3 to 37.0 ± 2.0 μg/L and was at least 1000-fold higher than the concentrations of OCPs and phenols (Table 1). Through the quantitative data from chemical analysis and TEQ calculation, the estimated TEQcals in waters for DBP ranged from 101.0 ± 5.4 to 239.9 ± 12.7 μg/L NH3 (Figure 2B), respectively. Based on the TEQ approach, it was revealed that dibutyl phthalate (DBP) accounted for 53.7 ± 8.2% to 105.5 ± 16.7% of TEQbio with a correlation coefficient of $r = 0.84$ ($p < 0.05$, Figure S3, Supporting Information), while diethylhexyl phthalate (DEHP) accounted for a little of TEQbio (<1%). It could be speculated that DBP may play the major role in the TR antagonistic activity in drinking water while DEHP also showed some contribution.

DBP and DEHP belong to a chemical family known as phthalates which are synthetic compounds widely used as polymer additives in plastics, polyvinyl chloride, rubber, cellulose, and styrene production to improve their softness and flexibility (*26*). In recent years, increasing evidence from in vivo and in vitro studies demonstrate that DBP and DEHP can affect the thyroid system. In a TR reporter gene in vitro assay using a recombinant *Xenopus laevis* cell line, DBP and DEHP were reported to exhibit T_3 (*27*). Several in vivo studies in laboratory animals indicate that the function of the thyroid is a target of phthalate plasticisers that may cause thyroid hyperactivity (*28*−*30*). For humans, free testosterone (fT) was significantly and negatively correlated with urinary levels of DBP and DEHP (*31*). Many studies found phthalates were rapidly metabolized, and most of the phthalates and their metabolites were cleared from the body within a few days (*32, 33*). But one should consider that even transient disruption of normal thyroid homeostasis will lead to disastrous outcomes, especially in the developing nervous system (*5*). And what is worse, DBP and DEHP can readily cross the placenta and are developmental and reproductive toxicants in laboratory animals (*34, 35*). At present, we have no reliable information about whether the level of TR antagonistic activity in drinking water can affect human thyroid hormone system. Considering that T_3 is always present in vivo ranging from 13.9 to 26.4 nM (*36*), it is suggested that ensuring the safe drinking water will be increasingly challenging.

Exposure to DBP and DEHP and other phthalates in the general population is widespread (*37*). Recently, phthalates were also detected in pooled breast milk samples from American women (*38*) and in infant formula (*39, 40*). The same thing has also happened in China, where widespread pollution of DBP and DEHP and other phthalates in biological samples has been reported in recent years (*41, 42*). Due to the widespread application of phthalates, they have been the most abundant compounds in various environmental matrices compared to other organic contaminants in China (*43*). In Beijing, 2.0 μg/L of diisobutylphthalate was found in the finished drinking water (*18*). It was also reported that DBP and DEHP were found up to 3.8 and 6.5 mg/kg in the urban area of Beijing (*44*). In the present study, 15.6 μg/L DBP and 7.1 μg/L DEHP were detected in the raw water. The relatively high concentration of DBP and DEHP may be due to that sampling was conducted in a dry season (May) in Beijing. It was reported that DBP and DEHP were found up to 8.3 and 5.9 μg/L in the dry season in raw drinking water in Southern California whereas in wet season they were not detected (*45*). It was reported that coagulation, flocculation, and precipitation were largely ineffective for removing dissolved organic contaminants (*46*). Although activated carbon was reported to be effective for removing organic contaminants, in the present study it had no obvious removal effect for DBP and DEHP. It was reported that several compounds were detectable in effluent from active carbon filtration, and the removal efficiency using activated carbon was largely dependent on water quality (*47*). For the drinking waters in China, DBP was reported to 17.3 μg/L in Chongqing city and 76 μg/L in Hangzhou city in the finished drinking water, which were higher than the raw water (*48, 49*). In Southern California, 2.7 μg/L DEHP was found in the finished drinking water, which was little lower than the raw water (*45*). Thus the high concentration of DBP and DEHP in the finished water may be caused by contamination of source water in the dry season and low efficiencies of the treatment processes for DBP and DEHP removal.

In the literature survey, only one study was conducted to survey the TR agonistic activities in drinking water using PC-DR-LUC method and it found no agonistic activity (*50*). There was no work carried out on the survey of TR antagonistic activities in drinking waters. Ishihara et al. who used TR-mediated luciferase gene activation found strong antagonistic activities in water samples from paper manufacturing plants (PMPs) (*51*). Gutleb et al. (*52*) reported that sediment extracts showed TR antagonistic activities in the presence of T_3 using T-screen method. In conclusion, this report described for the first time the TR antagonistic activity throughout drinking water treatment processes, and in source water. DBP may be the major TR antagonist in the drinking water of Beijing. Treatment processes are not able to remove all TR antagonists, so good integrated management practices and technologies are needed to prevent further deterioration of our water sources by reducing thyroidal disruptive pollutants from entering our waterways.

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Supporting Information Available

Analytical details on analysis and bioassay confirmative results. This material is available free of charge via the Internet at http://pubs.acs.org.

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