T-screen and yeast assay for the detection of the thyroid-disrupting activities of cadmium, mercury, and zinc

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Abstract In the present study, a two-hybrid yeast bioassay and a T-screen were used to screen for the thyroid receptor (TR)-disrupting activity of select metallic compounds (CdCl₂, ZnCl₂, HgCl₂, CuSO₄, MnSO₄, and MgSO₄). The results reveal that none of the tested metallic compounds showed TR-agonistic activity, whereas ZnCl₂, HgCl₂, and CdCl₂ demonstrated TR antagonism. For the yeast assay, the dose–response relationship of these metallic compounds was established, and the concentrations producing 20 % of the maximum effect of ZnCl₂, HgCl₂, and CdCl₂ were 9.1×10^{-5} , 3.2×10^{-6} , and 1.2×10^{-6} mol/L, respectively. The T-screen also supported the finding that ZnCl₂, HgCl₂, and CdCl₂ decreased the cell proliferation at concentrations ranging from 10^{-6} to 10^{-4} mol/L. Furthermore, the thyroid-disrupting activity of metallic compounds in environmental water samples collected from the Guanting Reservoir, Beijing, China was evaluated. Solid-phase extraction was used to separate the organic

extracts, and a modified two-hybrid yeast bioassay revealed that the metallic compounds in the water samples could affect thyroid hormone-induced signaling by decreasing the binding of the thyroid hormone. The addition of ethylenediaminetetraacetic acid (30 mg/L) could eliminate the effects. Thus, the cause(s) of the thyroid toxicity in the water samples appeared to be partly related to the metallic compounds.

Keywords In vitro bioassay · Toxic metals · Thyroid receptor · Thyroid-disrupting activity

Introduction

Heavy metals are widely dispersed in the environment and are a major concern in the modern industrial context of pollution because they tend to bioaccumulate (Denier et al. 2009). Heavy metals have the ability to cause detrimental health effects by affecting reproduction, fertility, as well as the immune and neuronal systems, and by leading to carcinogenesis (Shiue 2015; Martins et al. 2014). In the last 15 years, the potential health threat posed by endocrine-disrupting chemicals (EDCs) has come to the forefront of toxicology and represents a major concern among the public community (Buha et al. 2013). Several studies based on in vitro and in vivo assays have revealed that heavy metals, such as cadmium (Cd), mercury (Hg), and zinc (Zn), act as potential EDCs by activating estrogen receptor (ER) and modulating the estrogenic activity of endogenous hormones (Denier et al. 2009; Isidori et al. 2010; Ali et al. 2010). However, the endocrine disruption induced by chemicals is not restricted to the sex hormone system but also includes thyroid hormone (TH) disruption (Murk et al. 2013).

Thyroid hormones participate in several important physiological processes, including the regulation of energy metabolism, growth and differentiation, and the development and

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maintenance of brain function (Murk et al. 2013). However, increasing evidence from animal and in vitro studies indicates that the thyroid is vulnerable to endocrine-disrupting effects (Boas et al. 2012). Industrial chemicals characterized as thyroid-disrupting chemicals (TDCs) include polychlorinated biphenyls (PCBs), dioxins, brominated flame retardants (BFRs), pesticides, perfluorinated chemicals (PFCs), phenols, phthalate esters (PAEs), and perchlorate (Boas et al. 2012; Shi et al. 2009).

Although the research focus has mainly been placed on organic compounds, metallic compounds, particularly heavy metal compounds, are starting to be studied for their ability to disrupt the TH signaling pathways of various living organisms (Curčić et al. 2012; Hammouda et al. 2008). For example, Zn is required for the proper function of 1,5'-deiodinase, which is the enzyme required for the conversion of thyroxine (T_4) into triiodothyronine (T_3). Another metal, Cd, has been reported to inhibit thyroid function and induce thyroid dysfunction (Buha et al. 2013; Gupta et al. 1997; Gupta and Kar 1999). Furthermore, there is literature data supporting the hypothesis that the direct influence of toxic heavy metals, for example manganese (Mn), copper (Cu), and Zn, on thyrocytes plays a role in thyroid cancer etiology (Yaman et al. 2007).

The effects of THs depend on signal transduction via the binding of nuclear thyroid receptors (TRs), which subsequently interact with thyroid-response elements (TREs) in the promoter regions of genes to either enhance or inhibit gene transcription (Yen et al. 2006). However, the available data on the thyroid-disrupting effects of heavy metals mediated by the interaction with the TR are limited. A previous study reported that Zn plays a role in T_3 binding to TR and is crucial for the binding of TR to its target genes (Wada and King 1986; Surks et al. 1989). Zn-chelation enhances the ability of T_3 to induce target gene expression in rat pituitary tumor cells (GH₃) (Sciaudone et al. 2000). Moreover, Lukaski et al. (1995) found an inverse relationship between the TR binding of T_3 and the Cu. Taken together, all of these findings suggest that heavy metals may disrupt TR-mediated transcription and affect the thyroid system.

Thus, investigations of the heavy metals that may disrupt TR-mediated transcription should be promoted in order to resolve some of the environmental threats to human health. The objective of the present study was to employ a T-screen and a two-hybrid TR yeast assay to evaluate the thyroid disruption caused by selected heavy metals. The selected metals, namely Cd, Zn, Hg, Cu, Mn, and magnesium (Mg), are common pollutants in the environment and major sources of concern for both human and wildlife health (Fu et al. 2014). Environmental water samples were incorporated in this study because heavy metals are widely dispersed into the aquatic environment and thereby form a source of exposure for organisms in aquatic food chains.

Materials and methods

Chemicals

3,3',5-Triiodo-L-thyronine (T_3 , 95 %) and dimethylsulfoxide (DMSO, 99.5 %) were purchased from Sigma Chemical (St. Louis, MO, USA). Amiodarone hydrochloride (AH) was purchased from Shanghai Pharmaceutical (Shanghai, China). HPLC-grade dichloromethane, hexane, and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Cadmium chloride ($CdCl_2$), zinc chloride ($ZnCl_2$), mercury chloride ($HgCl_2$), cupric sulfate ($CuSO_4$), manganese sulfate ($MnSO_4$), and magnesium sulfate ($MgSO_4$) were obtained from Sigma-Aldrich. Stock solutions for all of the compounds were prepared at final chemical concentrations of 0.02 mol/L as reported by Denier et al. (2009). Seven concentrations of chemical, ranging from 2×10^{-9} mol/L to 2×10^{-3} mol/L, were obtained by serial dilutions.

Sample collection and processing

Environmental water samples were collected in September 2012 from the Guanting Reservoir in China. The locations of the eight sampling sites are shown in Fig. 1. Each of the water samples (4 L) was collected in separate amber glass bottle. The samples were stored at 4 °C prior to treatment. All of the samples were treated within 24 h.

The environmental water samples, procedure blanks (Milli-Q water, conductivity of 18.2 Ω), and control samples (a T_3 or T_3 + AH solution in Milli-Q water) were filtered through glass fiber filters (0.7 μm, Waterman, England) to remove insoluble materials. After filtration, volumes of 500 mL of the water samples were collected and stored at 4 °C in glass containers until used for the modified two-hybrid TR yeast assay. The other water samples, including the procedure blanks, were treated according to procedures described (Li et al. 2010). Solid-phase extraction was performed using a C18 cartridge (500 mg, Waters, USA) conditioned according to the manufacturer's directions. The cartridges were forced under vacuum at a flow rate of approximately 6 mL/min. After extraction, 500 mL of residual water and 500 mL of residual water added with ethylenediaminetetraacetic acid (EDTA, 30 mg/L) were also collected for bioassay using the modified two-hybrid TR yeast assay.

The conventional two-hybrid TR yeast assay

The bioassays, including the agonistic and the antagonistic activity tests, were used to evaluate the thyroid-disrupting activity of the selected heavy metal compounds, as described by Li et al. (2008). All of the experiments were performed in triplicate. Each assay group comprised the chemicals, the

Fig. 1 Sampling sites in the Guanting Reservoir

positive control (T_3 for agonistic activity or T_3 + AH for antagonistic activity), and the negative control (DMSO).

The yeast strain hTR-GRIP1 was grown overnight at 30 °C and 130 rpm with vigorous shaking. During the assay, exponentially growing overnight cultures were diluted with a synthetic dextrose/-Leu/-Trp (SD/-Leu/-Trp) medium to an OD_{600nm} of 0.75. The serial dilutions (5 μ L) of the chemicals were combined with 995 μ L of medium containing 5×10^3 yeast cells/mL, resulting in a test culture. To assess the antagonistic activities, the chemicals were tested in the presence of 2.5×10^{-7} mol/L T_3 , which produced a submaximal stimulatory response. The DMSO volume did not exceed 1.0 % of the total volume. Two hundred microliters of the test cultures were transferred into each well of a 96-well plate, and the plate was incubated at 30 °C with vigorous orbital shaking (800 rpm) on a titer plate shaker (Heidolph TITRAMAX 1000, Germany) for 2 h. The cell density of the culture was then measured using 600-nm irradiation (TECAN GENios A-5002, Austria). Fifty microliters of the test culture was transferred onto a new 96-well plate, and after the addition of 120 μ L of Z-buffer (disodium hydrogen phosphate dodecahydrate 21.51 g/L, sodium dihydrogen phosphate dehydrate 6.22 g/L, potassium chloride 0.75 g/L, and magnesium chloride heptahydrate 0.25 g/L) and 20 μ L of chloroform, the assays were carefully mixed (vortexed for 25 s) and pre-incubated for 5 min at 30 °C. The enzyme reaction was initiated by the addition of 40 μ L of *o*-nitrophenyl- β -D-galactopyranoside (13.3 mmol/L, dissolved in Z-buffer). The assays were

incubated at 30 °C for 60 min on a titer plate shaker. The reactions were terminated by the addition of 100 μ L of 1 mol/L Na_2CO_3 . Two microliters of the supernatant were transferred to a new 96-well plate, and the OD_{420nm} was determined. The β -galactosidase activity was calculated according to equations described by Li et al. (2008).

The modified two-hybrid TR yeast assay

A rapid and easy bioassay without water sample extraction, concentration, or sterilization was developed by Li et al. (2014). Exponentially growing overnight cultures were diluted with SD/-Leu/-Trp medium to an OD_{600nm} of 0.75. Five-milliliter samples of the cultures were centrifuged at 1000 g for 5 min (Sigma Laborzentrifugen 2K15, Germany), and the supernatant was discarded. The yeast cells and 0.5 mL of $10 \times$ SD/-Leu/-Trp medium were added to 4.5 mL of the water samples to yield the test culture. For the antagonistic test, 12.5 μ L of 10^{-4} mol/L T_3 (dissolved in DMSO) was added to the test culture, and the volume of DMSO did not exceed 0.25 % of the total volume. The test cultures were incubated at 30 °C with vigorous orbital shaking (130 rpm) on a shaking incubator (HZQ-Q, China) for 2 h. Two hundred microliters of the test cultures were transferred into each well of a 96-well plate, and the cell density of the culture was measured under 600-nm irradiation (TECAN GENios A-5002, Austria). The β -galactosidase activity was tested as described in “The conventional two-hybrid TR yeast assay” section.

T-screen procedure

Rat pituitary tumor cells (GH₃, purchased from the Cell Center of Basic Medicine, Beijing, China) were cultured at 37 °C and 5 % (v/v) CO₂ in a humid atmosphere in Ham's F10 medium with HEPES (GibcoBRL, New York, USA) supplemented with 2.5 % fetal calf serum (Gibco, Australian) and 15 % horse serum (Gibco, New Zealand). Prior to the T-screen procedure, the cells were incubated for 48 h in serum-free DMEM/F12 medium (PCM medium) to deplete the cells of the thyroid hormones, as described by Sirbasku et al. (1991). The T-screen was performed as described previously (Gutleb et al. 2005) with minor modifications. The old serum-free PCM medium was removed, and the cells were collected from the culture flask. The cell density was counted, and the cells were diluted with PCM to a density of approximately 2500 cells/well. Two hundred microliters of the cultures were transferred into each well of a 96-well plate, and the plate was incubated for 24 h. The serial dilutions (5 µL) of the chemicals were combined with 995 µL of the PCM medium to yield the test medium. To assess the antagonistic activities, the chemicals were tested in the presence of 2.5×10^{-10} mol/L T₃, which produced a submaximal stimulatory response. After discarding the old medium, 200 µL of the test medium was transferred onto a 96-well plate. The plate was incubated for 96 h, and the test medium was replaced every 48 h. After exposure, the cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was removed and replaced with 50 µL of PBS (potassium dihydrogen phosphate 0.27 g/L, disodium hydrogen phosphate 1.42 g/L, potassium chloride 0.2 g/L, and sodium chloride 8.0 g/L) containing 0.5 mg/mL MTT, which had been pre-incubated for 4 h at 37 °C. The medium was then removed, and 150 µL of DMSO was quickly added to each well to extract the dye. After an additional 10 min at room temperature and rapid agitation on a microtiter plate shaker, the plate was transferred to a microplate reader (TECAN GENios A-5002, Austria) equipped with a 490-nm filter to measure the absorbance of the extracted dye.

Cytotoxicity

To ensure that the increased/reduced activities observed by the bioassay were caused by true agonistic/antagonistic responses and not by cytotoxicity, the viability of the cells exposed to chemicals at the maximum assay concentration was measured.

The yeast cells were placed as in the original assay and then exposed for 2 h to medium containing chemicals. The cell viability was then determined spectrophotometrically as the change in the cell density (OD_{600 nm}) in the assay medium. The results were rejected when the ratio (OD_{600 nm-exposure medium}/OD_{600 nm-blank medium}) exceeded 110 % or was less than 90 %.

GH₃ cells are not able to divide in PCM without T₃ but retain their basal activity. To determine the cytotoxicity, GH₃ cells were plated and cultured with the PCM-containing test chemicals for 96 h. The cell viability was then tested by the MTT assay as described previously. The concentrations of the test compounds that inhibited the basal viability of GH₃ cells cultured in PCM without T₃ were considered to be cytotoxic (Gutleb et al. 2005).

Data analysis

The median-effect concentration (EC₅₀) and the concentration reducing 20 % of the maximum effect (RIC₂₀) for the compounds for which a dose–response curve could be constructed were calculated. The dose–response curve was generated using a four-parameter logistic model based on the Marquardt-Levenberg algorithm (Sigmaplot 4.0®, SPSS, Chicago, IL, USA) (Rehmann et al. 1999).

Results

Cytotoxic effects of metallic compounds

To quantify the cytotoxic effects, we measured the survival of yeast cells after treatment with the six metallic compounds at the maximum assay concentration (10^{−4} mol/L). The results suggested that the yeast cell viability of the CdCl₂ and HgCl₂ samples was significantly different from that of the reference culture ($p < 0.05$). The percent growth inhibitions were calculated to be 11.8 and 15.8 % for CdCl₂ and HgCl₂, respectively, showing that these chemicals exerted cytotoxic effects on yeast cells (Fig. 2a). The other chemicals did not exhibit any cytotoxic effects, even at the maximum assay concentration.

After treatment of GH₃ cells with CdCl₂, ZnCl₂, and HgCl₂, we also observed an inhibition of cellular growth with CdCl₂ and HgCl₂ at 10^{−4} mol/L (Fig. 2b). HgCl₂ exhibited cytotoxic effects even at the 1/10 dilutions (10^{−5} mol/L).

TR ant/agonistic activity of metallic compounds in the yeast assay

The conventional two-hybrid TR yeast assay performed with CdCl₂, ZnCl₂, HgCl₂, CuSO₄, MnSO₄, and MgSO₄ revealed that no chemicals exhibited TR-agonistic activities (Fig. 3a). However, the co-administration of the test chemicals with 2.5×10^{-7} mol/L T₃, which has been shown to induce submaximal β-galactosidase expression, demonstrated that several of the chemicals suppressed β-galactosidase activity (Fig. 3b). This inhibition by ZnCl₂, HgCl₂, and CdCl₂ demonstrated a dose-dependent relationship. The RIC₂₀ values of ZnCl₂, HgCl₂, and CdCl₂ were 9.1×10^{-5} , 3.2×10^{-6} , and 1.2×10^{-6} mol/L, respectively. The other chemicals did not

Fig. 2 **a** Cytotoxicity of the tested metallic compounds on yeast cells. **b** Cytotoxicity of the tested metallic compounds on GH₃ cells. The cytotoxicity of the samples is represented as the percent inhibition activity. The values are presented as the averages $\pm$ standard error ($n = 3$)

inhibit the T₃-induced β -galactosidase activity at the highest concentration that did not show cytotoxicity. As a result, the RIC₂₀ of these chemicals could not be calculated.

TR ant/agonistic activity of metallic compounds in the T-screen

To validate the T-screen modifications, the T₃-dependent GH₃ cell proliferation was measured, as shown in the Supporting Information (Fig. S1). The results obtained with the MTT assay correspond to those obtained with the resazurin detection assay (Schriks et al. 2006), with only a slight and insignificant shift in the median-effect concentration (EC₅₀) of T₃ from 2.0×10^{-10} to 6.1×10^{-10} mol/L (Fig. S1). Consistent with other reports (Norman and Lavin 1989), AH antagonized the response induced by 2.5×10^{-10} mol/L T₃ (Fig. S2). AH alone had no effect (data not shown), but at concentrations up to 5×10^{-7} mol/L, the T₃-induced response was significantly decreased. Visible cytotoxicity was observed at 10^{-4} mol/L. The RIC₂₀ was calculated to be 1.7×10^{-6} mol/L, which is in good agreement with published results (Schriks et al. 2006).

The effects of the metallic compounds (ZnCl₂, HgCl₂, and CdCl₂) on thyroid signaling were evaluated, and TR agonism was not detected (data not listed). The joint action of metallic

compounds and T₃ on GH₃ cells was assessed. Eight concentrations (from 10^{-11} to 10^{-4} mol/L) of each compound were tested in combination with 2.5×10^{-10} mol/L T₃, which induced submaximal cell proliferation. The presence of ZnCl₂ in the incubating medium inhibited the T₃-induced cell proliferation in a dose-dependent manner (Fig. 4). The maximum inhibition (31.9 %) was found at a concentration of 10^{-4} mol/L ZnCl₂, which did not have a cytotoxic effect. The RIC₂₀ value of ZnCl₂ was calculated to be 3.1×10^{-6} mol/L. A dose-dependent inhibition of the T₃ thyroidicity was also observed with HgCl₂. The thyroidic activity was inhibited significantly with the tested HgCl₂ concentration of 10^{-6} mol/L. At higher concentrations, HgCl₂ was toxic to the GH₃ cells. Similarly, CdCl₂ also reduced the T₃-induced cell proliferation, and the maximal-inhibition activity (26.8 %) was observed at 10^{-5} mol/L. The RIC₂₀ values of these metallic compounds could not be calculated.

TR antagonistic activity of environmental water samples

The thyroid-disrupting effects of the water samples were detected by the modified two-hybrid TR yeast assay, revealing that all of the water samples displayed antagonistic TR activities that inhibited β -galactosidase expression (Fig. 5). The

Fig. 3 **a** The agonistic thyroid receptor (TR) activities of the tested metallic compounds were determined using the yeast assay. **b** The antagonistic thyroid receptor (TR) activities of the tested metallic compounds were determined using the yeast assay. The data represent the results from three independent experiments with three replicates each

blank samples did not disrupt the TR. Interestingly, after extraction, the thyroid inhibition activities of the residual water decreased (Fig. 5). The extraction could eliminate more than 61.9 % of the total effect produced by the TR antagonists. Several residual water samples (GR1, GR5, and GR6) significantly differed from the negative control ($p < 0.05$), suggesting that several antagonists may be inorganic chemicals and exist in the residual water samples. After the addition of EDTA (30 mg/L) to the residual water samples, the inhibition activities decreased significantly, and the TR antagonistic

activities for all of the water samples were eliminated, showing that some metals may contribute to the TR antagonistic activity.

Furthermore, the concentrations of Zn, Hg, and Cd in the water samples were detected. The results showed that the concentrations of Hg and Cd were lower than 10^{-9} mol/L. However, the concentrations of Zn ranged from 5.2×10^{-8} to 1.6×10^{-7} mol/L, and the average concentration was 7.9×10^{-8} mol/L (Supporting Information).

Fig. 4 The antagonistic activities of the tested metallic compounds were determined using the T-screen. The data represent the results from three independent experiments with three replicates each

Discussion

In vitro methods are valuable and important tools for screening environmental compounds that exhibit potential endocrine-disrupting effects. In the present study, Mg^{2+} and Mn^{2+} were not found to disrupt the specific binding of T_3 to TR. This result is supported by Surks et al. (1989), who also reported that the concentration dependence responses for Mn^{2+} and Mg^{2+} to inhibit T_3 binding to TR were not illustrated. Cu^{2+} was not found disrupting the activity by the two-hybrid TR yeast assay, but in the previous work, Cu^{2+} showed limited ability to dissociate T_3 from TR extracted from cultured cells (Surks et al. 1989). One reason for the difference

Fig. 5 The antagonistic thyroid receptor (TR) activities of the water samples with various treatments were determined using the modified yeast bioassay. The data represent the results from three independent experiments with three replicates each. C18: solid-phase extraction using a C18 cartridge. Ethylenediaminetetraacetic acid (EDTA): addition of 30 mg/L of EDTA

observed between the two bioassays could be the different metal bioavailabilities between the yeast and the extracts of nuclei.

The results of the two-hybrid TR yeast and T-screen assays performed in this study demonstrate the anti-thyroid activity of several divalent metals, including Zn^{2+} , Hg^{2+} , and Cd^{2+} . Of these metals, particular attention was paid to Zn^{2+} because Zn^{2+} is necessary for the formation of the zinc finger structures of nuclear receptors, which play an important role in binding to DNA (Berg 1990). Moreover, Surks et al. (1989) reported that Zn^{2+} also might play a role in T_3 binding to TR. Zn^{2+} can displace of T_3 bound to TR. The addition of ZnCl_2 resulted in a concentration-dependent inhibition of the binding of T_3 to TR and that the ZnCl_2 concentration of 8×10^{-7} mol/L resulted in a significant decrease in the specific binding of [^{125}I] T_3 by TR (Surks et al. 1989), which is consistent with our finding that ZnCl_2 significantly inhibited the T_3 -induced enzyme activity at the concentration of 10^{-6} mol/L, as determined by the two-hybrid TR yeast assay. The GH₃ cell line has been described as a model cell line to study the basic TH-dependent cell physiology (Hohenwarter et al. 1996). In the present study, we also found that ZnCl_2 inhibits T_3 -dependent GH₃ cell proliferation in the concentration range from 10^{-6} to 10^{-4} mol/L. A similar result was reported by Chattopadhyay and Freake (1998), who suggested that the addition of exogenous zinc (ZnSO_4 , 10^{-5} – 10^{-4} mol/L) has negative effects on the T_3 responsiveness in GH₃ cells. The latter study also showed that Zn inhibits the T_3 -induced expression of the target proteins, such as fatty acid synthase and malic enzyme, in a dose-dependent manner (Chattopadhyay and Freake 1998). Sciaudone et al. (2000) showed that the restriction of Zn^{2+} availability with chelators (diethylenetriaminepentaacetic acid or EDTA) enhanced the action of T_3 to induce target gene expression in GH₃ cells, and that this positive effect could be blocked or reversed by 4×10^{-5} mol/L Zn^{2+} . The result argued against a toxic and generalized effect of Zn^{2+} removal on the cells (Sciaudone et al. 2000). The inhibitory effect of Zn^{2+} on T_3 binding was also reported to be specific for TR and the Zn^{2+} induced increase in the rate of dissociation of T_3 from

TR, which may be due to the interaction of Zn^{2+} with one or more of the conserved cysteine or histidine residues located within or adjacent to T_3 -binding site of the receptor (Surks et al. 1989).

Cadmium appears to be the largest single contributor to autoimmune thyroid disease, not only because Cd appears to play a pivotal role in thyroid disease but also because it is extremely toxic and exerts toxic biological effects at lower concentrations (Goyer et al. 1995). A few studies have reported that Cd inhibits thyroid function in mammals, fishes, and birds (Gupta and Kar 1999). However, the available data on the thyroid-disrupting effects of Cd are limited. In the present study, we suggested that CdCl_2 inhibits the response induced by T_3 and had TR antagonistic activity based on the results of the yeast and T-screen assays. A previous study suggested that CdCl_2 (10^{-6} – 10^{-4} mol/L) presented a concentration-dependent inhibition of T_3 -binding to TR, which is parallel to that observed with ZnCl_2 , and the half-maximal inhibition occurred at approximately 2×10^{-5} mol/L CdCl_2 (Surks et al. 1989). This value is consistent with our findings from the two-hybrid assay and the T-screen assay. Surks et al. (1989) also suggested that Cd^{2+} , like Zn^{2+} , might displace T_3 -binding site and decrease the binding affinity of T_3 with TR.

In the present study, Hg showed different cytotoxicity to the yeast and GH₃ cells, which is likely due to the differential susceptibility of these cells to HgCl_2 . However, the results of the anti-thyroid activity test by the two-hybrid TR yeast assay, as well as the inhibition of the T_3 -induced cell proliferation, as tested by the T-screen assay, suggest that Hg^{2+} may interrupt the thyroid function by regulating the TH-receptor signaling pathway. Few studies have examined the relationship between HgCl_2 exposure and direct thyrotoxicity. It has been suggested that men subjected to long-term workplace exposure to Hg may have a higher concentration of Hg in the thyroid and the pituitary than in the liver or the kidney (Kosta et al. 1975). A previous study also showed a relationship between Hg exposure and TH imbalance (Dundar et al. 2006). Sin et al. (1990) reported that the serum T_4 and T_3 decreased significantly in HgCl_2 -treated mice. However, the possible

mechanisms underlying the toxic effects of Hg are also discussed, and the effects of exposure to Hg on human thyroid function still require further confirmation.

It is very interesting that Zn, Cd, and Hg are included in group IIB in the periodic table. It has been reported that the members of this group show patterns in its electron configuration, especially the outermost shells, which result in trends in their chemical behavior. All of these may help to understand their TR-disrupting activities. Although the TR-antagonistic activities of ZnCl₂, HgCl₂, and CdCl₂ were detected in the yeast and T-screen assays, several differences between these assays were evident. For example, the yeast assay gave a higher RIC₂₀ value of ZnCl₂ than the GH₃ cell line. This variation could be due to the differences in the test mechanism of the assays. The GH₃ cells constitutively express both TR isoforms, and the TH-induced proliferation of GH₃ cells was used as the basis for the T-screen. The cell proliferation is an entirely genomic TR-mediated mechanism (Freitas et al. 2011). However, the two-hybrid yeast cell was simply transferred with hTR β , and the yeast cell was stably transfected with a thyroid-hormone-response-element-regulated β -galactosidase reporter gene, enabling the detection of a thyroid receptor-specific endpoint.

Heavy metals are widely dispersed in the environment. Steady population growth and industrialization have led to an increase in metal pollutants in the aquatic environment. Metal pollutants are distributed between the overlying water and the sediment, and the sediment has become the sink of these pollutants. Metal pollutants are a source of grave concern because of their toxicity, persistence, and accumulation. Measuring the toxic effects of heavy metals in aquatic environments may be important to obtain information regarding their impact on organism and ecosystem health. In the present study, pollutant extraction was performed, and the water samples collected from the Guanting Reservoir significantly inhibited the T₃-induced β -galactosidase activity. After the addition of EDTA, the inhibition was eliminated. The water samples have been shown to contain various heavy metals, such as Zn, Cd, and Hg, and the concentrations of Zn were equal or slightly lower than the lowest-observed-effect concentration. Although the major thyroid hormone-disrupting metals in the water samples have not yet been identified, these results are very promising for further research on the presence of thyroid hormone-disrupting metals in environmental water samples.

Conclusion

In summary, a two-hybrid yeast assay and a T-screen assay were utilized to identify the TR-agonistic and antagonistic activities of metallic compounds and water samples collected from the Guanting Reservoir. Our results suggest that ZnCl₂,

HgCl₂, and CdCl₂ inhibit the T₃-induced responses and may have anti-thyroid activity. Moreover, the metallic compounds in the water samples may affect TH signaling by decreasing the binding of the standard TR ligand (T₃). In future studies, the chemical forms of the heavy metals in the water samples will be analyzed to identify the major forms of the thyroid hormone-disrupting metals.

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