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Thyroid Hormones Receptor/Reporter Gene Transcription Assay for Food Additives and Contaminants

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ABSTRACT. Many of thyroid hormones disrupting chemicals induce effects via interaction with thyroid hormone and retinoic acid receptors and responsive elements intrinsic in target cells. We studied thyroid hormones disrupting effects of food additives and contaminants including BHA, BHT, ethoxyquin, propionic acid, sorbic acid, benzoic acid, CPM, aflatoxin B1, cadmium chloride, genistein, TCDD, PCBs and TDBE in recombinant HeLa cells containing plasmid construct for thyroxin responsive elements. The limit of response of the recombinant cells to T3 and T4 was 1×10^{-12} M. BHA, genistein, cadmium and TBDE were interacted with thyroid receptors with dose-responsive pattern. In addition, BHA, BHT, ethoxyquin, propionic acid, benzoic acid, sorbic acid, and TBDE showed synergism while cadmium chloride antagonism for T3-induced activity. This study elucidates that recombinant HeLa cell is sensitive and high-throughput system for the detection of chemicals that induce thyroid hormonal disruption via thyroid hormone receptors and responsive elements. Also this study raised suspect of BHA, BHT, ethoxyquin, propionic acid, benzoic acid, sorbic acid, TBDE, genisteine and cadmium chloride as thyroid hormonal system disruptors.

Keywords: Endocrine disruptor, Food additives, Food contaminants, Gene transcription assay, Thyroid hormones.

INTRODUCTION

Endocrine system such as pituitary, thyroid, gonads and etc. is very important in control of metabolism, development and growth of humans and other animals (Crisp *et al.*, 1998). Thyroid hormones, T3 and T4, synthesized in thyroid gland initiate biological reaction via binding with specific intracellular receptors, so called thyroid hormones receptor (THR) and retinoic acid receptor (RxR), and then the complexes are located to the DNA recognition site, thyroid hormones responsive elements, and induce transcription of responsive genes (Pham *et al.*, 1992; Danzo, 1997; Roy *et al.*, 1997). Thyroid hormonal system is very important for the regulation of overall metabolism in most tissues and growth and maturity of reproductive system (Crisp *et al.*, 1998). Recently, thyroid hormonal system has been more focused because of its systemic homeostatic functions on our bio- and eco-sphere. Some food additives like antioxidants and preservatives, pesticides and environmental contaminants that can be resided in foods are suspected as endocrine disruptors and their risks to human health pose mainly through daily food intake (Arnold *et al.*, 1996; Nilsson, 2000). There are several *in vitro* tests including receptor binding assay and GH3 cell proliferation assay to facilitate the process of identification and characterization of various thyroid hormones disrupting effects of chemicals (Lazar, 1990). Among *in vitro* tests, transformed cell lines expressing constructs of responsive elements containing reporter gene have been introduced as direct, sensitive and high-throughput tools for the screening thyroid hormones disruptors (O'Conner *et al.*, 2001). In addition, they provide information of chemical agonistic or antagonistic properties (Gutendorf and Westendorf, 2001). This study was performed to identify endocrine disrupting effects, especially on thyroid hormonal systems, of some food additives and contaminants using transformed mammalian cell line on the basis of receptor/

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CPM, chlorphyriphos-methyl; TCDD, 2,3,7,8-tetrachlorodibenzo dioxin; PCBs, polychlorinated biphenyls; TBDE, 2,2'4,4'-tetrabromodiphenyl ether; T3, triiodothyronine; T4, thyroxine.

reporter gene assay and to understand the properties of interaction of these chemicals with thyroid hormone receptors and responsive elements.

MATERIALS AND METHODS

Chemicals

Thyroxine (T4), triiodothyronine (T3), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), ethoxyquin, propionic acid, sorbic acid, benzoic acid, aflatoxin B1, zearalenone, cadmium chloride and genistein were obtained from Sigma Chemical Co. (St. Louis, MO). 2,3,7,8-tetrachlorodibenzo dioxin (TCDD) and polychlorinated biphenyls (Arochlor 1254, PCB) were obtained from Chem Service Inc. (Wester Chester, PA). Chlorpyriphos-methyl was obtained from Riedel-deHaen (Seeize, Germany) and 2,2',4,4'-tetrabromodiphenyl ether (TBDE) from Cambridge Isotope Laboratories, Inc. (Andover, MA). Luciferase assay kit was obtained from Promega Com. (Madison, WI) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cellular transfection

HeLa cell line transfected with plasmid construct of promoter sequences of thyroid hormone responsive elements ligated into thymidine kinase promoter and luciferase reporter gene was made. The plasmid construct was kindly provided from OSI pharmaceuticals Inc. (Uniondale, New York). Transformed HeLa cell line was used for the determination of chemical interaction with thyroid hormone receptors, retinoic acid receptors and thyroid hormone responsive elements (Barettino *et al.*, 1994; Wagner *et al.*, 1995; Glass, 1996; Bogazzi *et al.*, 1997).

Thyroid hormone receptor/reporter gene transcription assay

Transfected HeLa cells (5×10^4/ml medium) were cultured in 200 μl/well of Dulbecco's Modified Eagles Media (DMEM; Invitrogen Com., Carlsbad, CA) containing 1% charcoal-dextran-treated fetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin in 96 well culture dish for 16 hours. And then cells were treated with each concentration of T3, T4 or test chemicals solved in dimethyl sulfoxide (DMSO) for 24 hours. DMSO was added to cell culture media with 0.1% volume concentration. After treatment, the cells were washed with cold phosphate-buffered saline (pH 7.0) and then luciferase activity in the cells was measured with luciferase assay kit using microplate luminometer (Berthold Gmbh & Co., Badwildbad, Germany). For removing of T3, T4 and other steroid compounds from fetal bovine serum, charcoal-dextran was treated following to the protocol of Körner *et al.* (1998). For competitive binding test of each compound, 100nM of each compound was co-administered with 10^{-3} to 10 nM of T3 to the cells and then treated as above.

Calculations and statistics

Data were expressed as mean ± SE. Comparisons between vehicle control and chemical-treated group were performed one-way analysis of variances followed by Duncan's multiple comparison tests. In thyroid hormone receptor/reporter gene transcription assay, mean values of fluorescence intensity at each sample concentration were compared with that of corresponding vehicle control and expressed as mean percentage value of control ± SE.

RESULTS

Interactions with thyroid hormones receptors and thyroid hormone elements

HeLa cells transfected with plasmids expressing thyroid responsive elements were so sensitive to T3 and T4 that the limits of responsiveness were 10^{-12} M which is corresponding to 0.7~0.8 pg/ml culture media. T3 activated luciferase more highly than T4 as the concentration increased over 10^{-11} M, and the difference in maximum induction between T3 and T4 was around 30%. Antioxidants such as BHA, BHT and ethoxyquin showed 20~30% higher enzyme activities compared to vehicle control at 1 nM and increased weakly to approximately 1.5 times at 0.1 mM BHT and ethoxyqiun while increased more than 2 times by 0.1 mM BHA. Slightly high inductions were observed by propionic acid and benzoic acid compared to control without typical dose-response patterns. Sorbic acid showed peak induction as 1.8 times higher than control at 10 μM and then decreased at higher concentration. Cadmium, genistein and TBDE activated luciferase highly than vehicle control and the maximum induction by genistein was higher than T3 (Fig. 1).

Effects on T3-induced luciferase activation

Effects of test compounds on T3-induced luciferase reporter gene transcription were investigated to know their agonistic or antagonistic properties. When T3 from 5×10^{-4} to 10 nM was co-treated with 100 nM T4, luciferase activity was reached to peak value of 250% from 10^{-3} nM T3 and then remained peak induction. BHT, BHA, ethoxyquin, propionic acid, benzoic acid, sorbic acid, genistein and TBDE showed synergistic

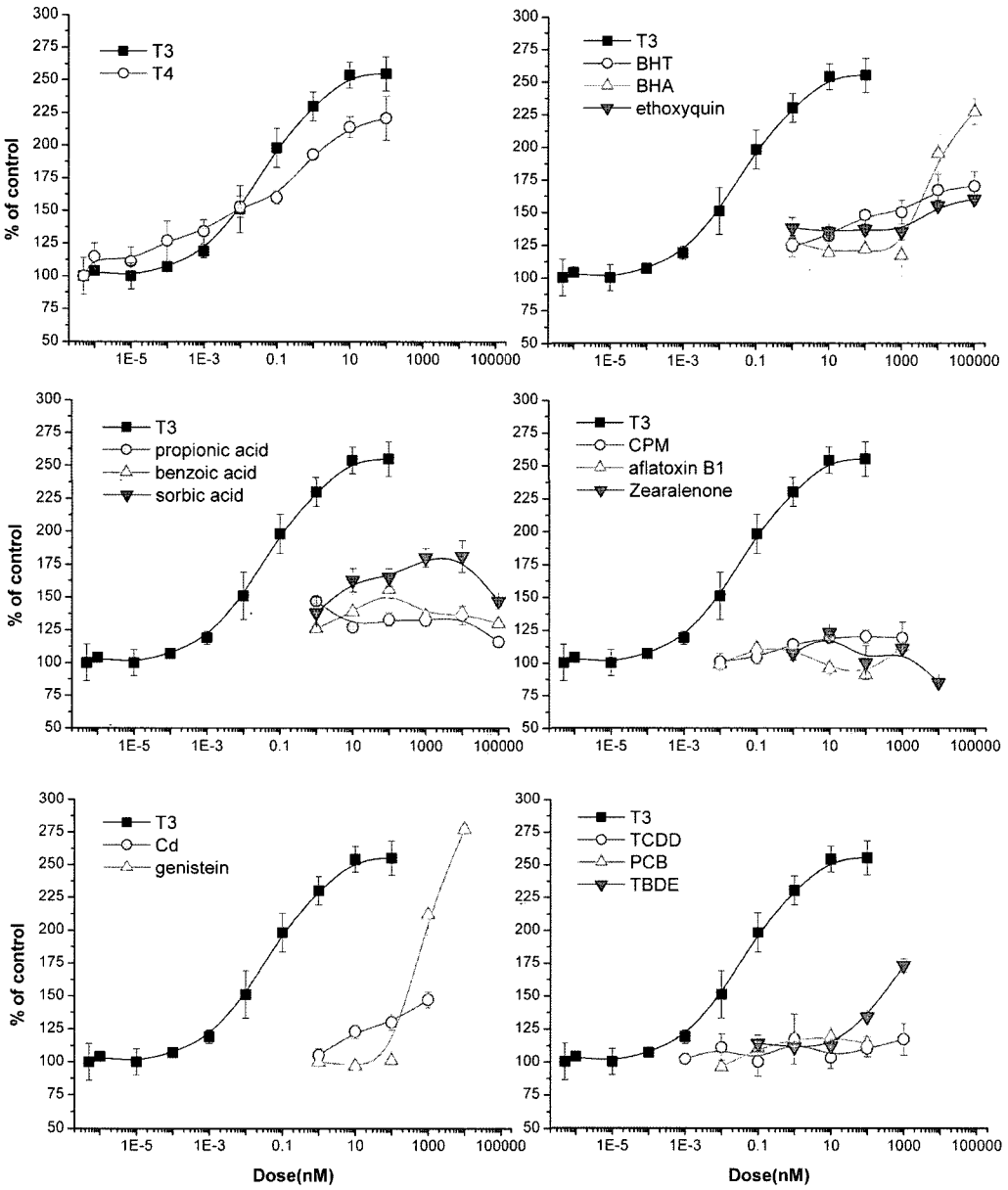

Fig. 1. Luciferase induction activity of each food additives and contaminants in thyroid hormone sensitive HeLa cells. Transfected HeLa cells were exposed to each test compound for 17 hrs. Values are mean percentages of control ± SE of three experiments each with 3 replicates.

effects on T3-induced reporter gene transcription while cadmium showed antagonistic effect. That is, cadmium decreased enzyme activity induced by T3 when they were co-treated even though cadmium increased luciferase activity when only it was treated. Other compounds had no effects on T3-activated luciferase induction (Fig. 2).

DISCUSSION

The identification of endocrine disrupters with understanding their action properties largely depends on empirical testing. Recently, USA EPA noticed that tests of endocrine disruption effects of chemicals should be expanded to cover androgenic and thyroid hormonal system in addition to estrogenic system to understand the risks of endocrine disruptors more sufficiently (Daston *et al.*, 2003). A large number of pesticides, industrial chemicals and phytoestrogens possessing hormone-like activities are distributed in all around environment including food chain (Roy *et al.*, 1997). Therefore, simple, sensitive, and specific *in vitro* assays which allow

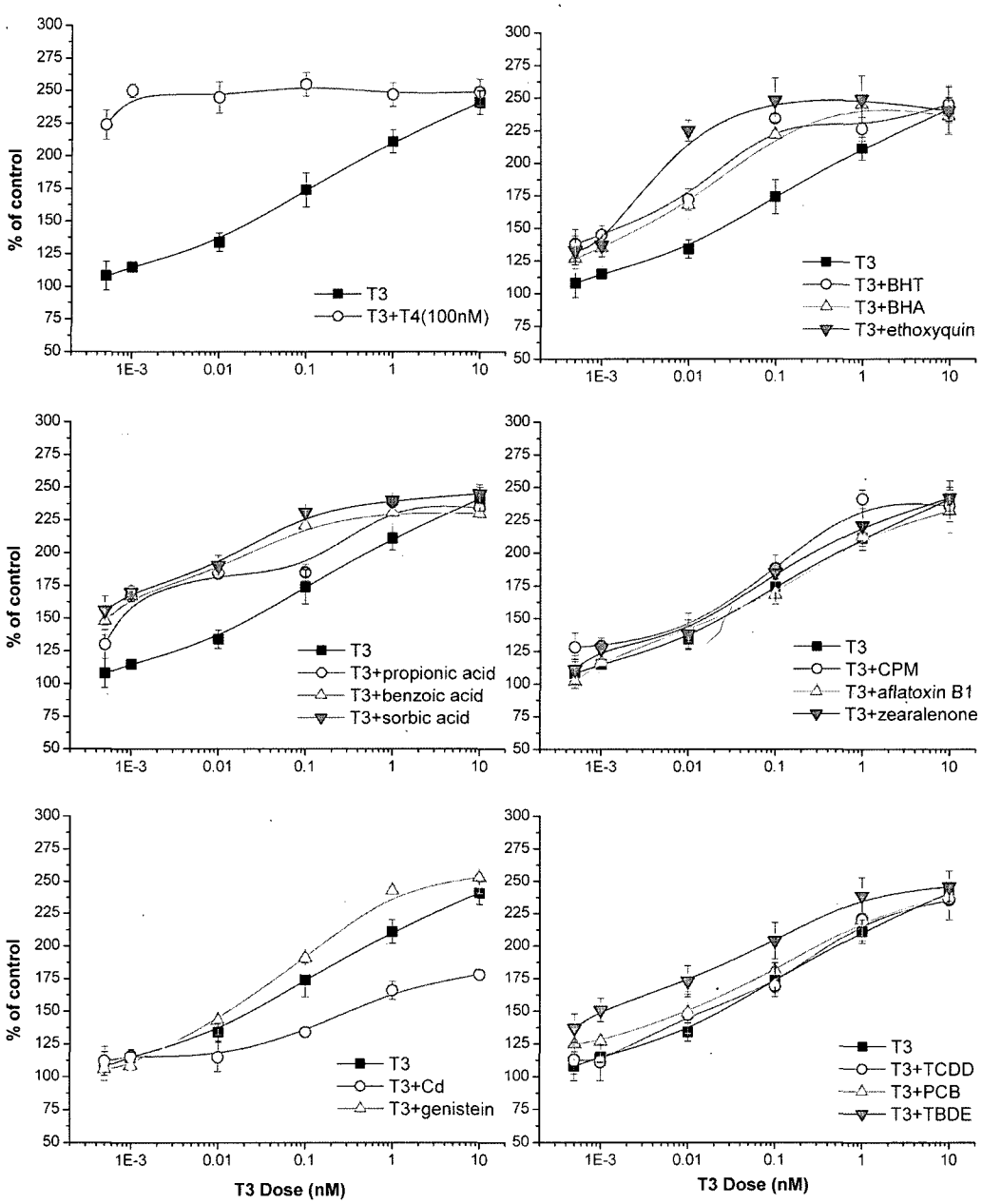

Fig. 2. Effect of each food additives and contaminants on T3-induced luciferase activities in thyroid hormone sensitive HeLa cells. 100 nM of T4 or test compounds were co-treated with 5×10^{-4}10 nM of T3 to the cells for 17 hrs. Values are mean ±SE of three experiments each with 3 replicates.

rapid screening of a large number of chemicals and environmental samples for possible hormone-like properties are required more than ever. Recombinant receptor/reporter gene assays are highly specific and sensitive system and they also reflect directly the interactions with receptors. Furthermore, the reporter gene assay may be adaptable to prescreening procedure for their high throughput screening process and no requirement of experimental animals (Takeyoshi *et al.*, 2002). However there are some limitations that the reactivity of system depends on the number of receptors, response elements, types of reporter gene and characters of the host cell type and etc. (Gray *et al.*, 1997). We used HeLa cell line as host cell of plasmid expressing thyroid responsive element for HeLa cell expresses well a lot of thyroid and retinoic receptors (Yamada-Okabe *et al.*, 2005). Thyroid hormone sensitive cells showed the limit of response to T3 and T4 at 1×10^{-12} M. 10 μM genisteine induced luciferase activation almost same as or higher than maximum induction induced by T3 while

zearalenone showed no effects. Genistein also showed weak synergistic effect when 100 nM of it was co-treated with T3. Genistein was reported to reduce thyroid peroxidase activity as a protein tyrosine kinase inhibitor and associated with goiter in animals and humans (Peterson an Barnes, 1996; Doerge and Chang, 2002). We did not investigate the cross-reactivity of the transfected cells with steroid hormones such as estradiol, testosterone and progesterone. In addition, the specificity of the cells was not checked using TR-antagonist. Further studies are required to confirm the transfected HeLa cells as a TR-specific screening system.

Some *in vivo* studies showed no hypothyroidism even though inactivation of thyroid peroxidase by genistein was apparent (Chang and Doerge, 2000). It is generally recognized that the anti-thyroid effect of genistein is caused by reduction of thyroid peroxidase but the interaction with thyroid receptor was not assessed before our study. More studies need to be performed for the full understand of the effects of genistein on thyroid function.

Some phenolic antioxidants such as BHT and BHA are used to prolong the shelf life of foodstuffs and to reduce nutritional losses by retardation of oxidation process. BHA is reported as a developmental toxic agent and a suspected endocrine disruptor (Kang *et al.*, 2005; Jeong *et al.*, 2005; Sonnenschein and Soto, 1998). There has been reported that antioxidants such as BHA and ethoxyquin delayed the onset of thyroiditis in obese strain chicken (Bagchi *et al.*, 1990). Jeong *et al.* (2005) showed that BHA induced anti-thyroidal effects as hypothyroidism and lower sperm formation and motility in one-generation reproductive study. In the present study, BHT and ethoxyquin showed weak induction effects while BHA showed high induction at above 10 μM in thyroid responsive element-expressing HeLa cells. And all tested antioxidants showed synergistic effects when treated with T3. The information of the causes of thyroid receptor binding effect or synergistic effect of those antioxidants has not been sufficient yet, so more mechanistic studies focused on interactions with thyroid receptors are required.

In our study, sorbic acid activated reporter enzymes in thyroid hormone sensitive HeLa cells. Also, all three preservatives, sorbic acid, benzoic acid and propionic acid, added induction effects to T3-induced lucuferase activation. There has not been full information to explain the thyroid effects of these preservatives yet. However, we can guess the acidity or oxidative status influenced by the preservatives may impact on thyroid hormone reaction.

Aflatoxin B1 had no effects on thyroid hormone receptors while cadmium interacted weakly with thyroid hormone receptor and antagonized T3-dependent thyroid receptor activity in our study. Cadmium stimulates the formation of metallothionein and binds to it in liver and kidney and induces growth retardation, testicular degeneration and reduced serum T4 concentration (Osweiler, 1996; Pilat-MArcinkiewicz *et al.*, 2002). Our study suggests that the interactions of cadmium with thyroid hormone receptor may result in its developmental, reproductive and thyroid toxicity.

TCDD, PCB and PBDE are representative environmental contaminants and bio-accumulative compounds for their lipophilicity. TCDD is classified as an endocrine disrupter and induces breast cancer, endometriosis, thyroid tumor, reduction of serum T4 and increase of serum thyrotropin (Kohn *et al.*, 1996; Rier and Foster, 2002). PCBs decreased serum T4 but do not alter gonadotropin, TSH or testosterone in serum (Desaulniers *et al.*, 1997; Osius *et al.*, 1999). PBDE is structurally similar to thyroid hormones and it decreases serum T4 accompanied by induction of uridine diphosphate glucuronosyl transferase (UDPGT) with no impact on serum TSH (Zhou *et al.*, 2001). In our study, TCDD and PCBs (Arochlor 1254) did not interact with thyroid hormone receptors. PBDE showed interaction with thyroid receptors and synergism on T3-induced enzyme activity. Overall, PBDE was considered as a weak thyroid hormone receptor agonist by this study.

Conclusively, the results obtained in this study suggest that transformed HeLa cells provide quick and sensitive tools for screening endocrine disrupting effects of food chemicals, especially on thyroid hormonal systems and have strong potential to figure out chemical interactions with thyroid receptors. These results are useful to understand endocrine toxicities of antioxidants, genistein, zearalenone, cadmium and PBDE while more studies are needed to elucidate the interactions of food preservatives such as benzoic acid, sorbic acid and propionic acid on thyroid receptors.

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