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Steroid Hormone Receptor/Reporter Gene Transcription Assay for Food Additives and Contaminants

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ABSTRACT. Many of endocrine disrupting chemicals induce effects via interaction with hormone receptors and responsive elements in target cells. We investigated endocrine disrupting effects of some food additives and contaminants including BHA, BHT, ethoxyquin, propionic acid, sorbic acid, benzoic acid, CPM, aflatoxin B1, cadmium chloride, genistein, TCDD and PCBs in yeast transformants expressing human steroid hormone receptors along with steroid responsive elements. The response limit of genetically recombinant yeast to 17β-estradiol, testosterone and progesterone was 1×10^{-16}, 1×10^{-12} and 1×10^{-13} M, respectively. BHT induced weak transcriptional activity in estrogen sensitive yeast, while BHA and sorbic acid interacted weakly with androgen receptor/responsive element. CPM induced transcriptional activities in all types of yeasts sensitive to steroid hormones. Zearalenone and genistein induced high transcriptional activation in estrogen sensitive yeast with relative potencies almost 10^8 folds lower than 17β-estradiol. TCDD induced transcriptional activation weakly in estrogen- and progesterone- sensitive yeasts. This study elucidated that recombinant yeast is a sensitive and high-throughput system and can be used for the direct assessment on chemical interactions with steroid receptors and responsive elements. Also, the present study raises the requirement of evaluation on the endocrine disrupting effects of BHT, BHA, sorbic acid, CPM and TCDD for their transcription activity in yeast screening system though weak in intensity.

Keywords: Endocrine disruptor, Food additives, Food contaminants, Gene transcription assay, Steroid hormone.

INTRODUCTION

Public concerns of chemicals surrounding our environment have arisen increasingly, because they may affect human health by disrupting normal function of endocrine system. Endocrine system including pituitary, thyroid, gonads and etc. is very important in the control of metabolism, development, and growth of humans and other animals (Crisp *et al.*, 1998). Hormones synthesized in endocrine glands initiate biological reactions in their target cells via binding with specific intracellular receptors followed by location of the hormone-receptor complexes on the DNA recognition site, called hormone responsive elements, and then finally induction of the transcription of responsive genes (Pham *et al.*, 1992; Danzo, 1997; Roy *et al.*, 1997). Chemicals inducing steroid hormone-like activity can lead to alteration of reproductive function, infertility, endometriosis, and cancers of reproductive organs (McLachlan, 1993).

Generally, food additives are recognized as safe or reasonably free of risks to human health. However, according to National Toxicology Program rodent bioassay, more than 40% of food chemicals, additives, and related chemicals were reported to be carcinogenic in one or more rodent groups though data were various according to dose, genotype, or route of administration (Johnson, 2002). In addition, food additives like BHT and BHA were classified as suspected endocrine disruptors (IEH, 1999). Some pesticides and environmental contaminants that can be reside in foods are also suspected as endocrine disruptors and their risks to human health pose mainly through daily food intake (Arnold *et al.*, 1996; Nilsson, 2000). Natural components of human diet are also considered as a potential source of endocrine disruptors especially for diets con-

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CPM, chlorphyriphos-methyl; TCDD, 2,3,7,8-tetrachlorodibenzo dioxin; PCBs, polychlorinated biphenyls.

tain rich of phytoestrogens (Daston *et al.*, 2003).

Many *in vitro* tests have been developed to facilitate the process of identification and characterization of various endocrine disrupting effects of chemicals (Baker, 2001). Among *in vitro* tests, transformed cell lines expressing receptor or reporter gene constructs have been suggested as direct, sensitive and high-throughput tools for the screening endocrine disruptors (O'Connor *et al.*, 2002). 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 steroid hormonal systems, of some food additives and contaminants using transformed yeasts on the basis of receptor/reporter gene assay and to understand the properties of interaction of these chemicals with steroid hormone receptors and responsive elements.

MATERIALS AND METHODS

Chemicals

17β-estradiol, progesterone, BHT, BHA, ethoxyquin, propionic acid, sorbic acid, benzoic acid, aflatoxin B1, zearalenone, cadmium chloride and genistein were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone (androsten-17β-ol-3-one) was purchased from TCI Inc. (Tokyo, Japan). 2,3,7,8-Tetrachlorodibenzo dioxin (TCDD) and polychlorinated biphenyls (Arochlor 1254, PCBs) were obtained from Chem Service Inc. (Wester Chester, PA, USA). Chlorpyriphos-methyl was obtained from Riedel-deHaen (Seeize, Germany). Oxalyticase was purchased from Enzogenetics Inc. (Corvallis, OR) and luciferase assay kit from Promega Com. (Madison, WI, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Yeast transformants

A strain of yeast (*Saccharomyces cerevisae* YPH500: MATα, ura^-, lys^-, ade^-, trp^-, his^-, leu^-) was used as a host transfected with following plasmids. An expression plasmid which contains the CUP1 metallothionein promoter fused to the human estrogen receptor cDNA and a reporter plasmid carrying two estrogen response elements upstream of the structural gene for β-galactosidase were inserted into host yeast to make estrogen-sensitive recombinant yeast. An expression plasmid which contains the CUP1 metallothionein promoter fused to either the human progesterone or androgen receptor, a reporter plasmid carrying two copies of a progesterone/androgen responsive element upstream of the structural gene for β-galactosidase and a plasmid encoding for SPT3 which enhances transcriptional efficacy in the androgen receptor assay were inserted into host yeast to make androgen-sensitive yeast. And for progesterone-sensitive yeast, a plasmid encoding for RSP5 which enhances transcriptional efficacy in progesterone receptor assay was used instead of SPT3 (Vegeto *et al.*, 1992; Gaido *et al.*, 1997) and other plasmids were same to androgen sensitive yeast. Those plasmids were kindly provided from Dr. Donald P. McDonnell (Department of Pharmacology, Duke University Medical Center, Durham, NC). Transformation of intact yeast cell was performed following to lithium acetate transformation protocol (Ausubel *et al.*, 2000). Transformed yeast cells were isolated using specific amino acid-deficient media plate (selective media plate) and then cultivated for the assessment of chemical interactions with human steroid hormone receptors and responsive elements.

Steroid hormone receptor/reporter gene transcription assay

Yeast transformants in each selective media were cultured overnight at 30°C with vigorous orbital shaking at 280 rpm until OD_{600} of the culture reached approximately to 1.2. 50 μl of yeast cell culture was added into 96-well microplate which already containing 50 μl of selective medium supplemented with 50 μM $CuSO_4$ and each standard steroid hormone as 17β-estradiol, testosterone and progesterone or test chemicals. Each test chemical was solved, and diluted in methanol, and then added to medium with 0.1% volume concentration. After measuring cell density at OD_{600} on plate reader, yeast transformants let to be responded to each chemical at 30°C for 4 h by shaking. After incubation, 100 μl of room temperature β-galactosidase assay buffer (10 μl of 10% sodium dodecyl sulfate solution, 2.7 μl of β-mercaptoethanol, 10 μl oxalyticase (200 unit/μl) per 1 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, pH 7.0) was added immediately after reading of OD_{600} and the culture well plate was incubated at 30°C. When reaction turned yellow, 50 μl of stop solution (2 M Na_2CO_3) was added to each well. OD_{420} and OD_{550} were measured on plate reader. β-galactosidase activity was calculated for Miller's units as;

$$1{,}000 \times \{OD_{420} - (1.75 \times OD_{550})\}/(t \times v \times OD_{600})$$

where t for reaction time (min) and v for volume of culture.

Calculations and statistics

Data were expressed as mean ± SE. Comparisons

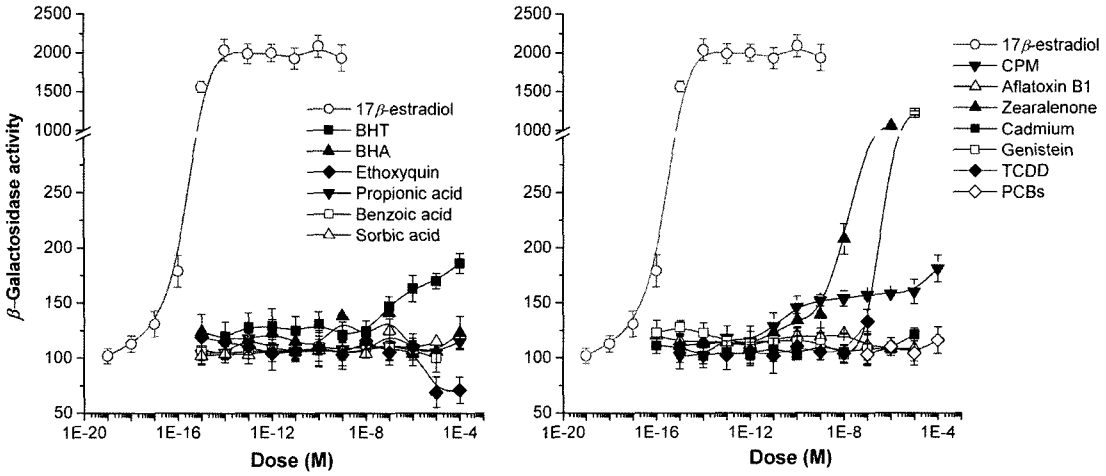

Fig. 1. Activity of each food additives and contaminants in estrogen sensitive yeast transformants. The values are mean ± SE and are obtained from three experiments each with triplicate wells.

between vehicle control and chemical-treated group were performed one-way analysis of variances followed by Duncan's multiple comparison tests. In steroid hormone receptor/reporter gene transcription assay, the induction effect (IE) of each chemical was calculated as;

IE = β-galactosidase activity maximum (compound)/ β-galactosidase activity (vehicle control)

The relative induction effect (RIE) was calculated as;

RIE (%) = {IE (test compound) - 1}/ {IE (each steroid hormone) - 1} × 100

Relative potency of each test compound was expressed as steroid hormone equivalency factor (estrogen equivalency factor, EEF; androgen equivalency factor, AEF; progesterone equivalency factor, PEF) and was calculated as;

EEF, AEF or PEF = EC50 (β-estradiol, androgen or progesterone)/ EC50 (test compound)

EC50 of each compound was obtained by sigmoidal fitting the semi-logarithmic dose-response curve using nonlinear regression algorithms provided by Origin software (version 6.0). Fitting was performed after fixing the upper limit as the value of maximal response induced by each steroid hormone (or each compound) and lower limit as that induced by vehicle control.

RESULTS

Interactions with estrogen receptor and responsive elements

Yeast transformant expressing human estrogen receptor and responsive elements showed high and specific responsiveness as 19 fold induction of β-galactosidase at 10^{-14} M 17β-estradiol compared to vehicle control. Significant induction was observed from 10^{-17} M, and EC50 obtained from sigmoidal curve fitting was 5.8×10^{-16} M. BHT activated β-galactosidase significantly from its concentration higher than 10^{-6} M. Relative induction effect of BHT was 2.15% compared to that of 17β-

Table 1. β-Galactosidase induction effects of 17β-estradiol and food additives and contaminants exposed to yeast transformants expressing estrogen receptors

Chemicals	IE	RIE (%)	EC50 (M)	EEF
17β-estradiol	19.2 ± 1.29*	100	5.80E-16	1
BHT	1.39 ± 0.07*	2.15	1.33E32	4.36E-48
BHA	1.14 ± 0.01	0.77	-	-
Ethoxyquin	0.97 ± 0.07	-	-	-
Propionic acid	1.11 ± 0.14	0.66	-	-
Benzoic acid	1.04 ± 0.12	0.22	-	-
Sorbic acid	1.23 ± 0.11	1.27	-	-
CPM	1.77 ± 0.12*	4.24	1.34E3	4.33E-19
Aflatoxin B1	0.95 ± 0.06	-	-	-
Zearalenone	8.05 ± 0.17*	38.8	1.54E-6	3.77E-10
Cadmium	1.09 ± 0.05	0.50	-	-
Genistein	9.91 ± 0.22*	49.1	6.44E-6	9.01E-11
TCDD	1.30 ± 0.11*	1.65	6.70E-7	8.66E-10
PCBs	1.13 ± 0.12	0.72	-	-

Induction effect (IE), relative induction effect (RIE), half effective concentration (EC50) and estrogen equivalency factor (EEF) were obtained by calculation or applying the fitting method described under Materials and Methods. In process of fitting to get EC50 of each chemical, the upper limit was fixed as maximum activity induced by 17β-estradiol (2,087) and lower limit as that of vehicle control (109) and standard error was weighted. - : negative value of IE or out of confident level in EC50. *Significantly different at $p < 0.05$ from vehicle control.

estradiol and its EC50 was 1.33×10^{32} M at which concentration induction reached 50% of the maximal response of estradiol and its efficacy was 4.36×10^{-48} times that of 17β-estradiol. Ethoxyquin decreased enzyme activity at higher than 10^{-5} M. Zearalenone and genistein induced expression of β-galactosidase reporter gene almost half of that induced by 17β-estradiol with relative potency 3.77×10^{-10} and 9.01×10^{-11} folds, respectively. CPM and TCDD also increased β-galactosidase significantly but weakly from 10^{-13} and 10^{-9} M, respectively. Relative potencies of CPM and TCDD were 4.33×10^{19} and 8.66×10^{10} fold less active than estradiol, respectively (Fig. 1 and Table 1).

Interactions with androgen receptor and responsive elements

Yeast transformant expressing human androgen/progesterone receptor, SPT3 and progesterone/androgen responsive elements was sensitively and highly reactive with testosterone as 19.5 folds induction of β-galactosidase at 10^{-6} M, and significant reaction was observed above 10^{-12} M. EC50 was 2.14×10^{-9} M that was 10^{7} higher than that of 17β-estradiol in estrogen sensitive yeast. BHA, sorbic acid, CPM and TCDD of tested compounds showed significant induction compared to vehicle control as 1.41 times at 10^{-8} M BHA, 1.45 times at 10^{-5} M sorbic acid, 1.50 times at 10^{-8} M CPM and 1.30 times at 10^{-6} M TCDD. Each EC50 of BHA, sorbic acid and CPM was calculated as 2.03×10^{28}, 4.09×10^{9} and 7.52×10^{7} M by sigmoidal fitting that is 1.05×10^{37}, 5.23×10^{19} and 2.85×10^{17} fold lower than that induced by testosterone (Fig. 2 and Table 2).

Table 2. β-Galactosidase induction effects of testosterone and food additives and contaminants exposed to yeast transformants expressing androgen receptors

Chemicals	IE	RIE (%)	EC50 (M)	AEF
Testosterone	19.5 ± 0.83*	100	2.14E-9	1
BHT	1.23 ± 0.14	1.24	-	-
BHA	1.41 ± 0.13*	2.22	2.03E28	1.05E-37
Ethoxyquin	1.16 ± 0.12	0.86	-	-
Propionic acid	1.18 ± 0.13	0.97	-	-
Benzoic acid	1.21 ± 0.13	1.14	-	-
Sorbic acid	1.45 ± 0.16*	2.43	4.09E9	5.23E-19
CPM	1.50 ± 0.10*	2.70	7.52E7	2.85E-17
Aflatoxin B1	1.23 ± 0.10	1.24	-	-
Zearalenone	1.00 ± 0.05	0	-	-
Cadmium	1.29 ± 0.10	1.57	-	-
Genistein	1.13 ± 0.11	0.70	-	-
TCDD	1.30 ± 0.08*	1.62	-	-
PCBs	1.13 ± 0.08	0.70	-	-

Induction effect (IE), relative induction effect (RIE), half effective concentration (EC50) and androgen equivalency factor (AEF) were obtained by calculation or applying the fitting method described under Materials and Methods. In process of fitting to get EC50 of each chemical, the upper limit was fixed as maximum activity induced by testosterone (2,574) and lower limit as that of vehicle control (132) and standard error was weighted. - : negative value of IE or out of confident level in EC50. *Significantly different at $p < 0.05$ from vehicle control.

Interactions with progesterone receptor and responsive elements

Yeast transformant expressing human androgen/progesterone receptor, RSP5 and progesterone/androgen responsive elements were highly reactive to progesterone as 15.7 times induction of β-galactosidase at 10^{-7} M compared to vehicle and significant induction was

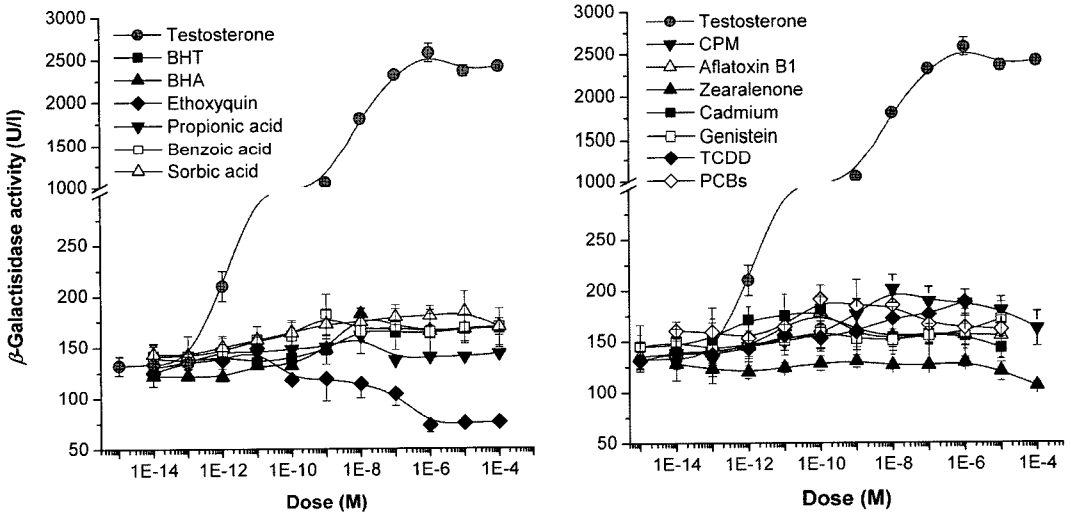

Fig. 2. Activity of each food additives and contaminants in androgen sensitive yeast transformants. The values are mean ± SE and are obtained from three experiments each with triplicate wells.

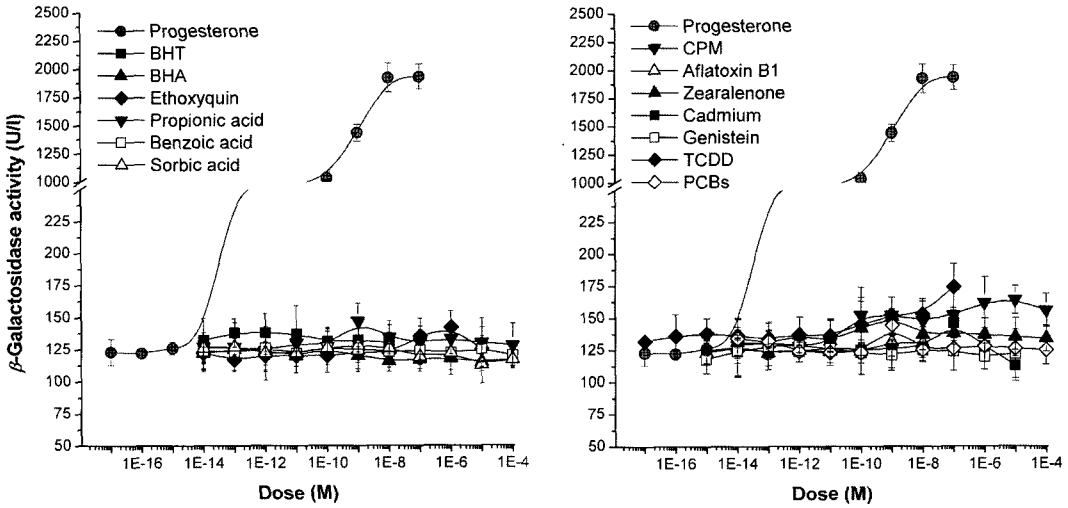

Fig. 3. Activity of each food additives and contaminants in progesterone sensitive yeast transformants. The values are mean ± SE and are obtained from three experiments each with triplicate wells.

observed from 10^{-13} M. EC50 was 1.35×10^{-11} M that is 10^5 times higher and 10^2 lower than that by 17β-estradiol in estrogen sensitive yeast and that by testosterone in androgen sensitive yeast, respectively. Only CPM and TCDD of tested compounds showed significant high β-galactosidase activity compared to vehicle control as 1.24 times at 10^{-5} M CPM and 1.32 times at 10^{-7} M TCDD. Each EC50 of CPM and TCDD was calculated as 92 M and 0.44 M, respectively by sigmoidal fitting that is 1.47×10^{13} and 3.07×10^{11} folds lower than that induced by progesterone (Fig. 3 and Table 3).

Table 3. β-Galactosidase induction effects of progesterone and food additives and contaminants exposed to yeast transformants expressing progesterone receptors

Chemicals	IE	RIE (%)	EC50 (M)	PEF
Progesterone	15.7 ± 0.83*	100	1.35E-11	1
BHT	1.04 ± 0.13	0.27	-	-
BHA	1.11 ± 0.10	0.75	-	--
Ethoxyquin	1.17 ± 0.11	1.16	-	-
Propionic acid	1.07 ± 0.10	0.48	-	-
Benzoic acid	1.03 ± 0.22	0.20	-	-
Sorbic acid	1.01 ± 0.14	0.07	-	-
CPM	1.24 ± 0.08*	1.63	0.92E2	1.47E-13
Aflatoxin B1	1.07 ± 0.16	0.48	-	-
Zearalenone	1.15 ± 0.11	1.02	-	-
Cadmium	1.18 ± 0.10	1.22	-	-
Genistein	1.09 ± 0.02	0.61	-	-
TCDD	1.32 ± 0.14*	2.18	0.44	3.07E-11
PCBs	1.08 ± 0.13	0.10	-	-

Induction effect (IE), relative induction effect (RIE), half effective concentration (EC50) and progesterone equivalency factor (PEF) were obtained by calculation or applying the fitting method described under Materials and Methods. In process of fitting to get EC50 of each chemical, the upper limit was fixed as maximum activity induced by progesterone (1,931) and lower limit as that of vehicle control (123) and standard error was weighted. - : negative value of IE or out of confident level in EC50. *Significantly different at $p < 0.05$ from vehicle control.

DISCUSSION

The identification of endocrine disruptors with understanding their action properties largely depends on empirical testing. Recently, USA EPA noticed that tests of endocrine disruption effects should expand to cover androgenic and thyroid hormonal effects in addition to estrogenic effects 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 distributing in all around environment including food chain (Roy *et al.*, 1997). Therefore, simple, sensitive, and specific *in vitro* assays that allow rapid screening of a large number of chemicals and environmental samples for possible hormone-like properties are required more than ever. In the recent years *in vitro* assays with different endpoints like recombinant reporter gene induction in yeast and mammalian cells, ligand binding to isolated hormone receptors, and proliferation of estrogen-sensitive human cells have been developed and applied (Klotz *et al.*, 1996; Körner *et al.*, 1998). Receptor binding assays are less sensitive than other test systems and serve limited information in distinguishing between receptor agonists and antagonists (Baker, 2001). Cell proliferation assay, one of the most widely used *in vitro* cell assays for the detection of estrogenic compounds, provides information of more direct biologi-

cal interactions with the estrogen receptor. However, the assay has problems with inter-laboratory variation according to cellular strains and culture conditions. Recombinant receptor/reporter gene assays are highly specific and sensitive system, and they also reflect direct interactions with receptors. Furthermore, the reporter gene assays are adaptable in pre-screen for their high throughput process and no requirement of experimental animals (Takeyoshi *et al.,* 2002). However, there are some limitation 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).

In our study, the response limit for 17β-estradiol is 10^{-16} M (2.7×10^{-5} pg/ml). This is 10^{6} orders of magnitude lower than other yeast reporter gene assay (Gaido *et al.*, 1997) and 10^{6-7} orders of magnitude lower than MCF-7 cell proliferation assay or transformed MCF-7 cell transactivation assay (O'Connor *et al.,* 1998), and approximately 10^{6-7} folds lower than physiological serum levels of E2 in women which are between 20 and 200 pg/ml depending on the menstruation cycle. Our progesterone- or androgen-sensitive yeasts were 10^{3} folds more sensitive than other results (Gaido *et al.,* 1997). The differences in reactivity of receptor/reporter gene assay are mainly coming from the number of receptors and responsive elements expressed in the systems, the types or strains of cells and culture condition. Gaido *et al.* (1997) used *Saccharomyces cerevisae* BJ3505 strain for the development of estrogen sensitive yeast and *Saccharomyces cerevisae* YPH500 for androgen or progesterone sensitive yeasts. We used *Saccharomyces cerevisae* YPH500 for three kinds of estrogen, androgen or progesterone sensitive yeasts. It is assumed the big difference in estrogen activity between laboratories is coming from the differences in host yeast strain. So, more strict standardized protocols are needed to narrow the inter-laboratory variances.

O'Connor *et al.* (1998, 2000) demonstrated that estrogen receptor agonist induces high transactivation in all estrogen-, androgen- and progesterone- receptor gene expressing yeasts with the order of estrogen- > androgen- > progesterone- sensitive yeast in responsiveness, while estrogen receptor antagonist or weak estrogen receptor agonist increases reporter gene activation only in estrogen sensitive yeast. Androgen receptor agonist induces high transactivation in androgen sensitive yeast and weak induction in estrogen- or progesterone-sensitive yeast, while androgen receptor antagonist just shows induction only in androgen sensitive yeast. Progesterone receptor agonists induce high activity only in progesterone sensitive yeast or concurrently weak induction in androgen sensitive yeast. In our study, zearalenone and genistein produced maximal responses in estrogen sensitive yeast comparable to half of that induced by 17β-estradiol with EC50 values of 1.54×10^{-6} and 6.44×10^{-6} M, respectively, but had no effect on androgen and progesterone sensitive yeast. Significantly high induction of β-galactosidase was observed when genistein was over 10^{-6} M and zearalenone was over 10^{-7} M. Genistein is mainly isolated from soy food products with biphasic properties that it is estrogenic at its concentration lower than 10^{-8} M but is antiestrogenic as its concentration increased (Wang *et al.,* 1996). Zearalenone, a β-resorcyclic acid lactones is well-known fungus estrogen found in *Fusarium* spp. that can be contaminated in food products and is classified as a weak estrogen with a potency two to four times less than that of estradiol. We can suppose genistein and zearalenone are weak estrogen receptor agonists though we did not perform competition binding assay.

Some phenolic antioxidants such as BHT and BHA are used to prolong the shelf life of foodstuffs and to reduce nutritional losses by retarding oxidation. BHA is reported as a developmental toxic agent and suspected endocrine disruptor for its affinity to estrogen receptor *in vitro* and induction of proliferation of MCF-7 cell line (Sonnenschein and Soto, 1998). In addition, BHA was reported to behave like an androgen antagonist in *in vitro* and short-term *in vivo* study (Kang *et al.,* 2004). Jeong *et al.* (2005a) showed that BHA induced anti-androgenic effect in rat one-generation reproductive toxicity study. BHT induced very weak reporter gene transcription only in estrogen sensitive yeast while BHA induced weak activity in androgen sensitive yeast, but their relative potencies were too low compared to 17β-estradiol or testosterone to suspect them as weak estrogen or androgen antagonists in the present study. So, chemicals induce weak responsiveness in yeast screening can not be overlooked for they have potency to induce endocrine disruption *in vivo* system.

In our study, sorbic acid activated reporter enzymes very weakly in androgen sensitive yeast among antifungal agents. It has not been reported for the endocrine disruption effects or reproductive toxicity of sorbic acid until now. The present study may give room to reconsider its safety.

CPM, a kind of organophosphate insecticides, showed weak induction effects in all of estrogen, androgen and progesterone sensitive yeast transformants with the highest induction in androgen sensitive yeast. We assume it as weak estrogen or androgen receptor agonist. CPM showed anti-androgenic effects in the Hershberger assay

and rat one-generation reproductive study (Jeong *et al.*, 2005b; Kang *et al.*, 2005). TCDD, PCBs and TBDE are representative environmental contaminants and bioaccumulative compounds for their lipophilicity. TCDD is classified as an endocrine disruptor and induces breast cancer and endometriosis (Kohn *et al.*, 1996). PCBs are also suspected to be estrogenic compound due to their chemical structure resembling that of DDT (Desaulniers *et al.*, 1997; Osius *et al.*, 1999). In our study, TCDD induced weak reporter gene transcription in estrogen and progesterone sensitive yeasts. However PCBs (Arochlor 1254) showed no effect on steroid receptors. Overall, TCDD is assumed as a weak estrogen receptor agonist or progesterone receptor agonist.

Conclusively, the results obtained in this study suggest that yeast transformants assayes give quick and sensitive indication for evaluation of endocrine disrupting effects of food chemicals and have strong potential to figure out chemical interactions with steroid receptors and responsive elements. These results may provide good and simple information on the endocrine toxicities of BHA, BHT, genistein, zearalenone, CPM and TCDD and to go forward for more complex *in vivo* study to elucidate the interactions of the compounds.

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