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Hormonal Effects of Several Chemicals in Recombinant Yeast, MCF-7 Cells and Uterotrophic Assays in Mice

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Abstract Many methods have been developed for screening chemicals with hormonal activity. Using recombinant yeasts expressing either human estrogen receptor [*Saccharomyces cerevisiae* ER + LYS 8127 (YER)] or androgen receptor [*S. cerevisiae* AR + 8320 (YAR)], we evaluated the hormonal activities of several chemicals by induction of β-galactosidase activity. The chemicals were 17β-estradiol (E2), testosterone (T), ρ-nonylphenol (NP), bisphenol A (BPA), genistein (GEN), 2-bromopropane (2-BP), dibutyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and butylparaben (BP). To assess the estrogenicity of NP, the result of the *in vitro* recombinant yeast assay was compared with an E-screen assay using MCF-7 human breast cancer cells and an uterotrophic assay using ovariectomized mice. In the YER yeast cells, E2, NP, BPA, GEN, and BP exhibited estrogenicity in a dose-response manner, while TCDD did not. All the chemicals tested, except T, did not show androgenicity in the YAR yeast cells. The sensitivity of the yeast (YER) assay system to the estrogenic effect of NP was similar to that of the E-screen assay. NP was also estrogenic in the uterotrophic assay. However, in terms of convenience and costs, the yeast assay was superior to the E-screen assay or uterotrophic assay. These results suggest that the recombinant yeast assay can be used as a rapid tool for detecting chemicals with hormonal activities.

Key words: Recombinant yeast, endocrine disrupter, hormone receptor, β-galactosidase activity, uterotrophic assay, E-screen assay

It has recently been suggested that the release of "endocrine disrupters (EDs)" into the environment has resulted in adverse health effects on wildlife populations and humans [16, 44]. Human sperm counts have declined significantly throughout the world during the past fifty years, which is a significant public health concern [4, 5]. In addition, the EDs persisting in the environment are known to disrupt normal endocrine systems of wildlife [6, 9, 13, 43, 44]. Some estrogenic chemicals bind to estrogen receptors [2], interfere with the binding of physiological ligands to steroid hormone-binding proteins [11, 28], and show immunotoxicity [36]. Such estrogenic compounds, structurally heterogeneous and nonsteroidal, were found in many places including the environment [15, 19, 46], food cans [3], experimental tools [23, 40], dental sealant [31], pharmaceuticals [17], and cosmetics [34].

To characterize the estrogenicity of chemicals, many screening methods have been developed. The uterotrophic assay in ovariectomized rats [42] or immature female rats or mice [29, 37] is one of the methods that has been used to detect the estrogenic activity of chemicals. In contrast, Kelce *et al.* [21] assessed the hormonal activities of chemicals in normal or castrated mature male rats. These *in vivo* methods are valuable for extrapolation to humans, because of the multiple mechanisms involved *in vivo*, such as absorption, metabolism, distribution, and excretion of chemicals. However, these methods are time consuming, labor intensive, and costly. In contrast, *in vitro* assays are quicker, cheaper, and can provide useful insights on the mechanism of hormonal action. The E-screen assay using estrogen-sensitive cells, such as MCF-7 cells, has been used for screening estrogenic chemicals [41, 45]. However, such experiments are vulnerable to the risks of contamination and also labor-intensive.

Since it was reported that recombinant yeast cells have human estrogen receptor functions [27], several estrogenicity screening assays have been developed using them [1, 8].

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The recombinant yeasts have two plasmids: an expression plasmid which contains a CUP1 metallothionine promoter fused to either a human estrogen or androgen receptor cDNA, and a reporter plasmid carrying an estrogen or androgen response element upstream of the structural gene of β-galactosidase. Recombinant yeast assays have merits due to the ease of manipulation and can process large numbers of samples, quickly and inexpensively.

Using recombinant yeasts expressing human estrogen receptor [*Saccharomyces cerevisiae* ER + LYS 8127 (YER)] or androgen receptor [*S. cerevisiae* AR + 8320 (YAR)], we evaluated the hormonal activities of several chemicals by the induction of β-galactosidase activity. These included 17β-estradiol (E2), testosterone (T), p-nonylphenol (NP), bisphenol A (BPA), genistein (GEN), 2-bromopropane (2-BP), dibutyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD), and butylparaben (BP). The estrogenicity of NP measured by the recombinant yeast assay was compared with the results of the E-screening assay using MCF-7 human breast-cancer cells. We also confirmed the results obtained from the *in vitro* assays in the uterotrophic assay using ovariectomized mice.

MATERIALS AND METHODS

Chemicals

NP was obtained from Kanto Chemical Co. Inc. (Japan), TCDD from GL Science Inc. (Japan), and 2-BP was from Fluka Chemie AG (Switzerland). E2, T, BPA, GEN, BP, DBP, DEHP, and 4-hydroxytamoxifen (OHT) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All the test chemicals were dissolved in appropriate solvents for each experiment.

Recombinant Yeast Cells

S. cerevisiae ER + LYS 8127 (YER) and *S. cerevisiae* AR + 8320 (YAR) were obtained from Dr. Donald P. McDonnell (Duke University Medical Center, U.S.A.). The yeast cells were stored in 20% glycerol at - 80°C. The YER cells were grown in a shaking incubator at 30°C with 200 rpm in a selective growth medium containing yeast nitrogen base (without amino acid, 67 mg/ml), 1% dextrose, L-lysine (36 µg/ml), and L-histidine (24 µg/ml). The selective growth medium for YAR was prepared from the YER selective growth medium supplemented with L-tryptophan (48 µg/ml) and adenine sulfate (40.8 µg/ml). The yeast cells were then allowed to grow until the OD values at 600 nm reached between 1.0 and 2.0.

Estrogen- and Androgen-Receptor Assays

The procedures were slightly modified from the method described previously [14]. Briefly, the yeast cells were diluted to an $OD_{600\,nm}$ value of 0.03 for YER and 0.06 for YAR, and then 5 µl of 10 mM $CuSO_4$ per ml of yeast cell medium was added. Five milliliters of the diluted yeast were treated with 5 µl of each test chemical in a 50-ml conical tube. As positive controls, E2 and T were used in the estrogen- and androgen-receptor assays, respectively. The tubes containing the yeast were incubated for 18 h in a shaking incubator at 30°C with 200 rpm. After incubation, the cultures were diluted in the appropriate selective growth media to produce an $OD_{600\,nm}$ value of 0.25. The diluted culture (100 µl) was added to three wells of a 96-well microtiter plate. β-Galactosidase activity was determined by the addition of 100 µl of a Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 2 mg/ml *O*-nitrophenyl-β-D-galactopyranoside (ONPG), 0.1% sodium dodecyl sulfate, 50 mM β-mercaptoethanol, and 200 U/ml oxalyticase (Enzogenetics, Cornavillis, U.S.A.). The $OD_{420\,nm}$ and $OD_{590\,nm}$ values of each well were measured using the Titertek Multiskan MCC/344 plate reader after allowing the tube to stand for 20 min. The $OD_{420\,nm}$ value of each well was corrected by subtracting the $OD_{590\,nm}$ value.

MCF-7 Cell Proliferation Assay

The protocol for the cell proliferation assay was described previously [39]. MCF-7 cells were grown in phenol red-free D-media (EMEM containing 50% increased amounts of all essential amino acids except glutamine, 50% increased amounts of all vitamins, and 100% increased amounts of all non-essential amino acids) supplemented with 5% fetal bovine serum (FBS) and 3 ml/l of a PSN antibiotic mixture (Gibco, U.S.A.). The cells were placed in an incubator maintained at 5% CO_2, 95% air and 100% humidity at 37°C. All compounds were then diluted with the phenol red-free D-media supplemented with 5% dextran-coated charcoal-stripped FBS (DCC-FBS; Hyclone, U.S.A.) and 3 ml/l PSN antibiotic mixture (test media). The DMSO concentration of the control media was less than 0.2%.

The cells (5×10^4) were plated in a 6-well culture plate (2 ml/well) in triplicate, and allowed to attach for 24 h. The phenol red-free D-media were replaced with phenol red-free D-media supplemented with 5% DCC-FBS, followed by incubation for 24 h, then the medium was removed and replaced by test medium (prepared as above) containing appropriate concentrations of compounds. The cells were incubated at 37°C for 3 days and the test media were changed once. The cells were then washed three times with phosphate-buffered saline and lysed with 1 ml of 0.1 N NaOH. The lysates were transferred into a 1.5-ml microcentrifuge tube and centrifuged for 2 min. The DNA content was determined by the method described previously [20]. The $OD_{260\,nm}$ value of the clear lysate was measured with a spectrophotometer (Du 650, Beckman, Fullerton, U.S.A.).

Animals

Seven-week-old female ICR mice were obtained from the Daihan Laboratory Animal Center (Korea). All animals were acclimatized for 1 week in the environmentally controlled room (temperature: 22±3°C, relative humidity: 55±5%, air-circulation frequency: 10- 12 times/h, artificial light: 150- 300 Lux from 7 a.m. to 7 p.m.). Healthy mice were ovariectomized (OVEX) under general anesthesia. The estrus cycle of OVEX mice were observed with daily vaginal smears for 2 weeks and the non-estrus cycling animals were used for the uterotrophic assay. The animals were housed in polycarbonate cages. Mice were fed mouse-rat pellets (Samyang Foods Co., Korea). The food and water were available *ad libitum.*

Uterotrophic Assay

The uterotrophic assay using OVEX mice was performed according to the method described previously [40, 42]. Five animals per group were subcutaneously injected for three consecutive days between 10:00- 11:00 a.m. with various doses of NP dissolved in corn oil or 17β-estradiol (10 μg/day/kg body weight). A total volume of 5-ml/kg-body weight was used. After determining body weights, the animals were killed by cervical dislocation 24 h after the final dose. The Uteri were excised, trimmed free of fat, pierced, and blotted to remove any excess fluid. The body of the uterus was cut just above its junction with the cervix and then weighed (wet weight). The uterus and vagina were fixed in 10% formalin, embedded in paraffin, sectioned at 3 μm, stained with hematoxylin and eosin, and photographed. Sections of uterus and vagina were observed under a light microscope, and the height of the luminal epithelium was measured.

Statistical Analysis

Data were analyzed using the SAS program for one-way ANOVA. If the overall F-test was significant, the two-tailed Dunnett's t-test was performed to determine significant differences at the level of $p<0.05$ or $p<0.01$ between means of the treatment and control groups.

RESULTS

Estrogenic Activity of Chemicals in Recombinant Yeast Assays

The sensitivity and the reproducibility of the YER yeast assay for estrogenic activity were evaluated using a natural steroid, E2 (Fig. 1). From a concentration of 1×10^{-10} M, E2 exhibited a significant increase of β-galactosidase activity ($p<0.05$). A dose-response increase in β-galactosidase activity in the YER yeast cells was also observed with NP, BPA, GEN, and BP, suggesting that they have estrogenicity (Fig. 1). NP caused a significant increase in the β-galactosidase activity from the concentration of 5×10^{-7} M, BPA from 1×10^{-6} M, GEN from 1×10^{-8} M, and BP from 5×10^{-7} M

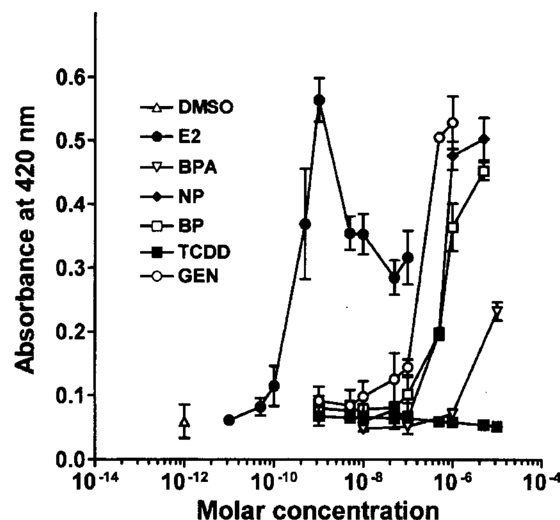

Fig. 1. Estrogenic activity of xenobiotics in the YER assay. Test chemicals were added to yeast cultures at concentrations ranging from 10^{-11} M to 10^{-5} M. The induction of β-galactosidase activity was determined at $OD_{420\,nm}$. Values represent the mean±SD of 3 separate experiments for each chemical.

(p<0.05). Compared to E2, the estrogenic potencies of NP, BPA, GEN, and BP were 1/5,000, 1/10,000, 1/1,000, and 1/5,000, respectively. TCDD at the concentrations used in this study did not show any significant increase in β-galactosidase activity (Fig. 1).

Androgenic Activity of Chemicals in Recombinant Yeast Assays

The sensitivity and the reproducibility of the YAR yeast assay for androgenicity were assessed using a natural

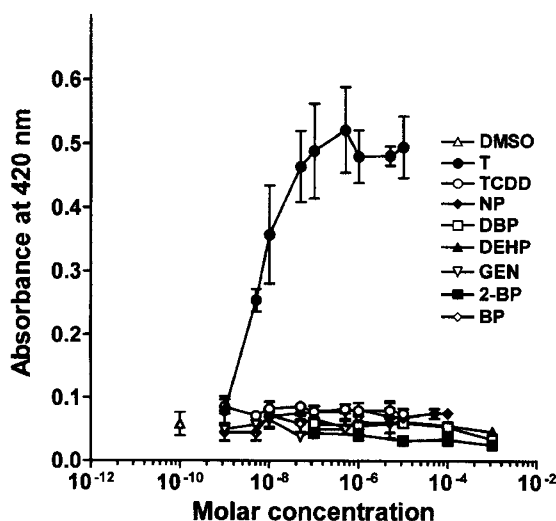

Fig. 2. Androgenic activity of xenobiotics in the YAR assay. Test chemicals were added to yeast cultures at concentrations ranging from 10^{-9} M to 10^{-3} M. The induction of β-galactosidase activity was determined at $OD_{420\,nm}$. Values represent the mean±SD of 3 separate experiments for each chemical.

steroid, T. From a concentration of 5×10^{-9} M, T increased β-galactosidase activity in a dose-dependent manner (Fig. 2). All the chemicals, including NP, 2-BP, TCDD, DEHP, DBP, GEN, and BP, did not increase β-galactosidase activity, indicating that they were not androgenic in the YAR yeast assay system (Fig. 2).

Effect of NP on MCF-7 Proliferation

An E-screen assay using MCF-7 cells was used for determining the estrogenicity of NP. The exposure of MCF-7 cells to NP increased their DNA content in a dose-dependent manner (Fig. 3). The DNA content of MCF-7 cells was mostly induced at a concentration of 1×10^{-6} M of NP, representing a 3.6-fold higher content than that of the control. Exposure to OHT at a concentration of 1×10^{-6} M inhibited the estrogenic effects of E2 and NP, as determined by the growth of the MCF-7 cells (Fig. 3). The growth inhibition caused by OHT was greater in NP than in E2.

Effects of NP on the Reproductive Tracts of OVEX Mice

The uterine filled with fluid was observed in 2 of 5 mice injected with E2, and in 1 of 5 mice injected with 1,000 mg NP/kg (data not shown). The body weights of the OVEX mice were not significantly different between the treatment groups. NP increased the uterine wet weight and the uterine wet weight/body weight ratio in a dose-dependent manner (Table 1). At a dose of 1,000 mg/kg, NP significantly increased the uterine wet weight by 2.2-fold and the ratio of the uterine wet weight *vs.* body weight by 2.28-fold, respectively ($p<0.05$). At the same dose, NP also significantly increased the number of uterine glands compared to the group which were injected with corn oil ($p<0.05$). Furthermore, NP caused increases in the uterine and vaginal luminal epithelial heights in a dose-dependent pattern. From a dose of 100 mg NP/kg, there were significant differences in the heights of the uterine and vaginal luminal epithelium between the control and the NP treatment groups (Table 1). At a dose of 1,000 mg NP/kg, the heights of the uterine

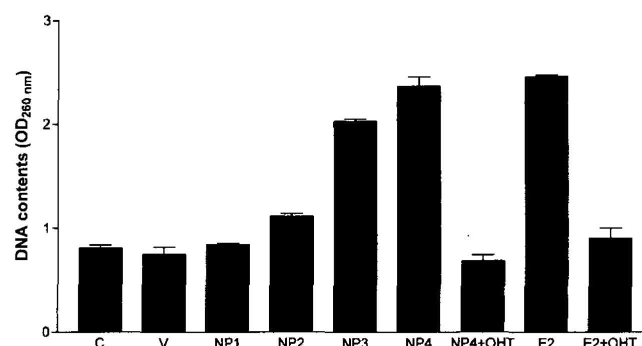

Fig. 3. Estrogenicity of p-nonylphenol (NP) and 17β-estradiol and its inhibition by 4-hydroxytamoxifen using an E-screen assay. 17β-estradiol (E2, 2.5×10^{-10} M) or NP was incubated with MCF-7 cells, and NP stimulated the MCF-7 cell growth in a dose-dependent manner but the treatment of 4-hydroxytamoxifen (OHT, 10^{-6} M) significantly inhibited the estrogenicity of E2 and NP (10^{-6} M). Values represent the mean±SD of 3 separate experiments for each chemical. C: media without hormones; V: DMSO; NP1: 10^{-9} M; NP2: 10^{-8} M; NP3: 10^{-7} M; NP4: 10^{-6} M; E2: 17β-estradiol, 2.5×10^{-10} M.

and vaginal luminal epithelium were about 2.4-fold and 5.6-fold higher than the control, respectively. Cornification of the vaginal epithelium was also observed at a dose of 1,000 mg NP/kg (Fig. 4).

DISCUSSION

A great number of chemicals, including environmental pollutants, detergents, pharmaceuticals, preservatives, natural compounds, and food ingredients, have been produced for diverse purposes. Some of them have been reported to have adverse health effects on humans and wildlife; for example, due to their hormonal activity [7]. However, many chemicals still need to be tested for their toxicity. By comparing a recombinant yeast assay with an *in vitro* E-screen assay and then validating the results with an *in vivo* uterotrophic assay, the present results demonstrated that a recombinant yeast assay system could be used to assess the hormonal activities of chemicals.

Table 1. Effects of p-nonylphenol on the reproductive tract of ovariectomized mice.

Group	Dose	Uterine wet weight (mg)	Uterus/body weight ratio	Number. of uterine glands	Height of uterine epithelium (μm)	Height of vaginal epithelium (μm)	Vaginal cornification
Corn oil		25.60± 2.45	0.076±0.008	19.89± 5.53	13.03±0.29	23.35±0.53	0/5
p-nonylphenol							
	0.1 mg/kg	22.80± 4.02	0.068±0.012	23.60± 8.64	13.50±0.25	23.60±0.48	0/5
	1 mg/kg	23.20± 1.18	0.071±0.008	27.89± 7.64	13.53±0.25	23.15±0.50	0/5
	10 mg/kg	26.18± 2.56	0.075±0.006	28.44± 7.86	13.95±0.26	22.50±0.36	0/5
	100 mg/kg	35.02± 8.58	0.097±0.016	32.22±11.08*	18.68±0.42**	48.60±1.16**	3/5[a]
	1,000 mg/kg	55.08±19.52**	0.173±0.057**	33.00±10.11**	31.55±0.48**	129.90±2.88**	5/5[b]
17β-estradiol							
	10 μg/kg	78.26±25.44	0.228±0.073	48.25±18.50	32.06±0.59	153.70±2.79	5/5[b]

17β-estradiol was used as a postive control; * denotes $p<0.05$; ** $p<0.01$; a, partial cornification; b, complete cornification.

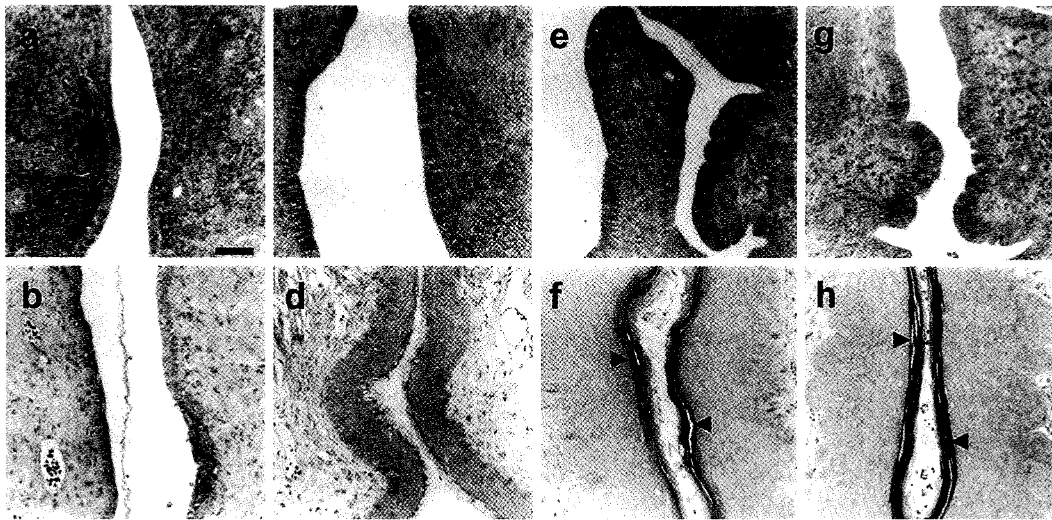

Fig. 4. Histological changes in the uterus of ovariectomized mice (a, c, e, and g) and in the vagina (b, d, f, and h) after treatment with various doses of NP for 3 days.
a, b, corn oil; c, d, NP 100 mg/kg; e, f, NP 1,000 mg/kg; g, h, E2 10 μg/kg. Both NP (1,000 mg/kg) and E2 induced cornification and sloughing of the vaginal epithelium (arrowheads). Bar size, 50 μm.

The YER yeast cells responded to E2, while the YAR yeast cells responded to T in a dose-dependent manner. Significant increases in β-galactosidase activity were induced at concentrations of 1×10^{-10} M of E2 and 5×10^{-9} M of T. The sensitivity of YER was similar to the result reported by Gaido *et al.* [14], however, the sensitivity of YAR was 1/10 of the previously reported data for dihydrotestosterone [38]. In the YER assay, the peak response at the E2 concentration of 10^{-9} M decreased until the E2 concentration reached 10^{-7} M, whereafter it increased again at higher E2 concentrations. The induction of β-galactosidase activity by T at a 10^{-6} M concentration decreased in the YAR cells, whereas it increased again at 10^{-5} M. The mechanism involved is still unknown.

NP, BPA, GEN, and BP were estrogenic in the YER assay, but none of the chemicals tested in the YAR assay (NP, GEN, BP, DEHP, DBP, TCDD, and 2-BP) showed any change of β-galactosidase activity. NP has been reported not to manifest any estrogen-like activity when measured at a dietary concentration as high as 2,000 ppm [10]; however, in this assay, estrogenicity was shown to be as low as 5×10^{-8} M of NP (about 11 ppb), which is in good agreement with previous *in vivo* and *in vitro* reports [10, 25, 30]. NP did not seem to bind to human androgen receptor (hAR). BPA was estrogenic in the YER assay, agreeing with the previously reported data [29]. The range of BPA concentrations, including the amount released from food cans, was enough to induce the proliferation of MCF-7 [3]. GEN is known to have biphasic effects of inhibiting breast cancer cell proliferation at high concentrations, by competing with estrogen for estrogen receptors, and can induce proliferation of cancer cells [12]. The current results showed that GEN significantly increased the β-galactosidase activity from a concentration of 1×10^{-8} M, however, concentrations over 10^{-6} M were toxic to the YER yeast cells. In the MCF-7 cell proliferation assay, the similar range of GEN has been reported to be estrogenic [18]. Although GEN prefers to bind to estrogen receptor β rather than estrogen receptor α, it does not seem to bind to hAR [24]. BP was effective in inducing β-galactosidase activity which was greater than that of BPA. BP is one of the paraben preservatives used in fats, oils, soaps, gums in hair shampoos and conditioners, skin care products, and other cosmetics [34]. Therefore, the products containing BP may be applied to the human body with continuous frequency and duration. There is no androgenicity of BP. TCDD has been reported to bind to the aryl hydrocarbon receptor and induce cytochrome P450 1A1 [32, 35]. Neither the YER nor the YAR yeast cells responded to the various doses of TCDD. This result indicates that TCDD is not likely to bind to hER or hAR. Although DBP and DEHP have been reported to be estrogenic [19, 47], in the present YAR assay, they did not change β-galactosidase activity when compared to DMSO, suggesting that DBP and DEHP are not androgenic. 2-BP was reported to induce amonorrhea, pancytopenia, and to elevate follicule-stimulating hormone levels in women. In men, it induces oligospermia and azoospermia, and reduces sperm mobility [22]. The mechanism involved has not been elucidated. In the YAR assay, there was no response with 2-BP. Accordingly, 2-BP is unlikely to manifest its toxic effects through hAR at the range of the concentrations tested.

Both the E-screen assay using MCF-7 cells and the uterotrophic assay using ovariectomized mice were used to assess the estrogenic activity of NP. The E-screen assay

was reported to be one of the more sensitive assay methods for screening estrogenic compounds [41]. In the YER assay, NP stimulated cell growth in a dose-dependent manner; an anti-estrogen compound, 4-hydroxytamoxifen, inhibited this growth. The maximal stimulatory effect was observed at a concentration of 1×10^{-6} M of NP. Estrogenic compounds have been reported to increase uterine weight and induce vaginal-cell cornification [26]. In the uterotrophic assay, the number of uterine glands or the heights of uterine- and vaginal-epithelia were more sensitive indication of estrogenicity by NP than the uterine wet weight. In comparing the effects on the uterine- and vaginal-epithelial growth, the height of vaginal luminal epithelium was more responsive than that of the uterine luminal epithelium. Therefore, NP was confirmed to be estrogenic, based on the complete cornification of vaginal epithelium.

Some chemicals, such as methoxychlor, fail to bind to hER while having an uterotrophic effect, whereas some chemicals bind to hER but fail to induce transcription or uterotrophy. In this regard, the recombinant yeast assays may be used as a pre-screening assay for the priority setting. In conclusion, the recombinant yeast assays expressing human steroid receptors are useful for screening hormonally active compounds, including natural hormones, several environmental chemicals, and phytoestrogens. Comparing the yeast assays with the E-screen assay or uterotrophic assay for the estrogenicity of nonylphenol, the yeast assays has several advantages such as easy manipulation, rapid screening, high sensitivity, and capability of dealing with a large number of samples. However, the characterization of the hormonal activities of certain chemicals should still be done by a combination of other *in vitro* and *in vivo* assays.

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