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Estrogenic Activity of Phthalate Esters by *In Vitro* VTG Assay Using Primary-cultured *Xenopus* Hepatocytes

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Estrogenic activity of phthalate esters in dental soft resins was evaluated with an amphibian system consisting of a vitellogenin (VTG)-detecting Enzyme-Linked Immunosorbent Assay and a primary-cultured hepatocyte assay using adult male *Xenopus laevis*. In particular, phthalate esters − Di-n-butyl phthalate (DBP), Butyl phthalyl butyl glycolate (BPBG), Benzyl butyl phthalate (BBP), and Benzyl benzoate (BB) − were investigated. Bisphenol A (BPA) was prepared for comparison with these chemicals, and 17β-estradiol (E2) was used as a positive control. The chemicals were diluted in dimethyl sulfoxide (DMSO) to obtain final concentrations ranging from 10^{-11} to 10^{-4} mol/l. BPA induced estrogenic activity at a concentration of 1.1×10^{-6} mol/l, while E2 showed at 4.1×10^{-11} mol/l. DBP, BBP, BB, and BPBG showed no estrogenic activity at concentrations between 4×10^{-7} mol/l and 1×10^{-4} mol/l. The latter result indicated that these phthalate esters might be metabolically transformed into non-estrogenic substances in *Xenopus* hepatocytes. Furthermore, this study demonstrated that through *in vitro* metabolism assessment, the estrogenic activity of chemical substances could be directly detected in terms of VTG secretion in primary-cultured *Xenopus* hepatocytes.

Key words: Sandwich ELISA, Estrogenic activity, Phthalate ester, Dental soft resin

INTRODUCTION

Soft resins are widely used for short-term temporary restorations after cavity preparation and as denture lining materials in dentistry[1]. Generally, they are imparted flexibility by a plasticizer additive[2]. The most available plasticizer in dentistry are the phthalate esters[3], largely because of their very low toxicity. However, recent environmental toxicology researches expressed concern that some phthalate esters were identified as EDCs that indicate estrogenicity[4−6].

Exposure to EDCs with hormone- or antihormone-like activity is one possible cause for the alleged decline in male reproductive health in humans and the increase in reproductive deficits in wildlife[7,8]. Biological effects of EDCs may occur at a lower concentration as compared with that required to induce cytotoxicity effects on local irritation. Against this background, it is feared that EDCs may have a detrimental effect on the living systems even with the ingestion of a very small quantity.

There is growing concern that phthalate esters may cause endocrine disruption − as indicated by some bioassays; however, the results are not conclusive yet. In some previous studies, the authors reported on the estrogenic activity of phthalate esters by MCF-7 cell proliferation assay, receptor binding assays, reporter gene expression assays using cultured cells and yeast cells, and a yeast two-hybrid assay[9−13]. Despite the usefulness of these methods in detecting estrogenicity, they have their limitations too.

For the detection and characterization of environmental chemicals with potential to disrupt the endocrine system, *in vivo* bioassays are developed by the Organization for Economic Co-operation and Development (OECD) Task Force on Endocrine Disrupters Testing and Assessment. On the other hand, *in vitro* tests are also play an important role because they can rapidly identify suspect compounds and most certainly, reduce the number of test animals. In this connection, risk assessment of EDCs has gradually been conducted in screening tests that used yeast and bacteria, such as estrogen receptor competitive binding assay and reporter gene assay based on estrogen-responsive promoters[14].

However, these *in vitro* assays − as screening tests − lack *in vivo* metabolites. For instance, environmental compounds may be metabolically transformed into estrogenic or non-estrogenic substances in the body or cells. Therefore, it is also necessary to examine the direct effects of these compounds on animals or cells in the screening tests.

With regard to the abovementioned point, *in vitro* primary hepatocyte assay detecting hepatic vitellogenin (VTG) as a biomarker might be useful

for screening the estrogenic and anti-estrogenic activities of chemicals. Recently, yolk precursor protein VTG has been widely used as an estrogenic biomarker. It is synthesized in hepatocytes and induced directly by estrogenic compounds. Thus, through *in vitro* assessment of metabolism in cultured primary hepatocytes, the biological activities of estrogens and xenoestrogens are well exemplified by detecting the synthesis of VTG in male oviparous vertebrates[15,16].

We have proposed VTG as an ideal biomarker in the screening of estrogenic activities of endocrine disrupters in a wide range of oviparous animals[17]. In the present study, we evaluated the estrogenic activity of phthalate esters in dental soft resins with an amphibian system consisting of VTG-detecting ELISA and primary-cultured hepatocyte assay using adult male *Xenopus laevis*.

MATERIALS AND METHODS

Specimens

For standard solutions, Di-n-butyl phthalate (DBP), Butyl phthalyl butyl glycolate (BPBG), Benzyl butyl phthalate (BBP), and Benzyl benzoate (BB) were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. Bisphenol A (BPA; Kanto Chemical Co., Tokyo, Japan) and 17β-estradiol (E2; Sigma-Aldrich Inc., St. Louis, MO, USA) were also investigated. The chemicals were diluted in dimethyl sulfoxide (DMSO; Kanto Chemical Co., Tokyo, Japan) to obtain the experimental concentrations.

Animals

Adult male *X. laevis* frogs were supplied by Yamamura Frog Store (Hiroshima, Japan). The animals were kept at 22℃ under a 12:12-hour light-dark cycle, and fed with *Xenopus* No. 3 (Oriental Yeast Co. Ltd., Tokyo, Japan) three times per week.

Primary culture

Hepatocytes were isolated from adult male *X. laevis* as described by Kawahara *et al.*[16]. A one-step perfusion method was used as follows. The liver was perfused with 100-200 ml of a perfusion solution (pH 7.4) containing 0.55% NaCl, 0.014% KCl, 1 mmol/l pyruvate, 0.05% glucose, 0.5% BSA, 10 mmol/l HEPES, and 0.1% collagenase (collagenase, Wako Pure Chemicals Co.). The perfused livers (about 2.5 g) were minced and incubated in the perfusion solution at 25℃ for 15 minutes with gentle shaking. Cell suspension was then sieved through a nylon mesh. Following which, the cells were washed four times with the culture medium by low-speed centrifugation at 300 rpm/min for 1-2 minutes, which eliminated the non-parenchymal cells almost totally from the final preparation (usually more than 95% liver parenchymal cells). The cells (4×10^4 cells) were then inoculated into 96-well tissue culture plates (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) and incubated in air at 22℃. The culture medium consisted of 50% Leibovitz L-15 medium which contained 1 μg/ml insulin, 10 nmol/l dexamethasone, 0.05% glucose, and antibiotics (50 U/ml of penicillin and 50 μg/ml of streptomycin).

After two days of preculture, the cells were exposed to estrogen by replacing the culture medium with an estrogen-containing medium. Estrogens to be tested were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 0.1 and 1 mmol/l and used after appropriate dilution with the culture medium (not to exceed 0.1% DMSO). 0.1% DMSO was used as a control. Various concentrations of phthalate esters in conjunction with physiologically relevant concentrations of E2 and BPA were tested. In brief, all chemicals were serially three-fold diluted in culture medium. The concentrations used were: 1×10^{-8} to 1.4×10^{-11} mol/l for E2, 1×10^{-5} to 4.1×10^{-8} mol/l for BPA, and 1×10^{-4} to 4.1×10^{-7} mol/l for the four phthalate esters. Culture medium was renewed every three days by replacing 2/3 volume of the medium. At the end of culture period, the culture media were harvested into 96-well microtiter plates.

Sandwich ELISA

VTG level in each culture medium was measured using *Xenopus* VTG ELISA Kit (Japan EnviroChemicals Ltd., Osaka, Japan). Albumin (ALB) in culture medium was measured with ELISA assay, according to our previous report[17]. Reactions were carried out at room temperature, and absorbance of reactions was determined at 450 nm with a microplate reader (Spectra Fluor, Tecan, Salzburg, Austria). Assay range of the standard curves was 0.25-50 ng/ml for VTG and 0.25-250 ng/ml for ALB. VTG concentration in culture medium was calculated from the linear part of the VTG standard curve. Culture media were diluted at least twice for ELISA assay.

Statistical analysis

Statistical analysis was performed using the software package SPSS 11.5J (SPSS Inc., Chicago, USA). VTG concentration in culture medium was expressed as mean ± standard deviation of the mean for three replicate wells within the same isolation. Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison tests. Differences were considered significant at $p<0.05$.

RESULTS

Typical ELISA standard curves used to quantify Xenopus VTG and ALB

Assay range of the standard curves was 0.25-50 ng/

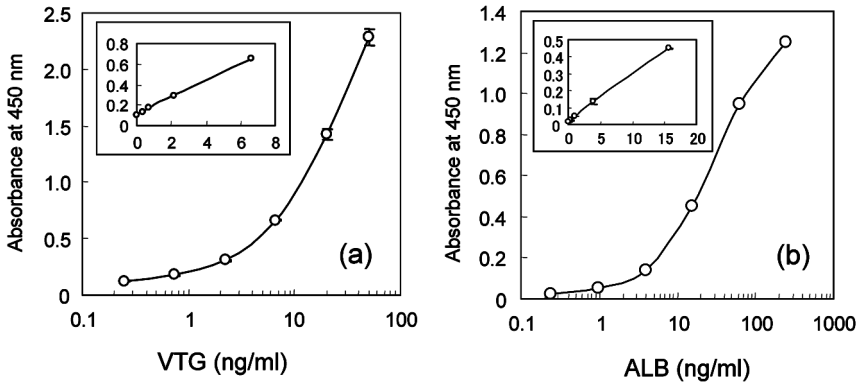

Fig. 1 Standard curves of ELISA for quantifying (a) VTG and (b) ALB. The inserts show locally magnified curves. Each value indicates mean±S.D. (n=3).

ml for VTG and 0.25-250 ng/ml for ALB (Fig. 1). A linear increase (0.2-6 ng/ml) in the ELISA reaction was observed for VTG concentrations. These ELISA standard curves were used as the calibration curves for the chemicals tested. Culture media were diluted at least twice for ELISA assay. Therefore, the quantitation limit of VTG in the culture medium was 0.5 ng/ml.

Dose-response curves of BPA-dependent VTG and ALB induction

Dose-response curves of BPA-dependent VTG and ALB induction were compared to those of E2 (Fig. 2). VTG level of control was 0.53±0.06 ng/ml. BPA showed induction of VTG at a concentration of 1.1×10^{-6} mol/l (induced VTG level: 1.62 ng/ml), while E2 showed at 4.1×10^{-11} mol/l (induced VTG level: 3.91 ng/ml). Estimated values for the potency relative to E2 were obtained using the concentrations of E2 and BPA required for inducing 1 ng/ml VTG. It was estimated as 0.003% for BPA when E2 was 100% (Table 1).

Dose-response curves of phthalate esters

Dose-response curves of phthalate ester (DBP, BPBG, BBP, BB)-dependent VTG and ALB induction are shown in Fig. 3. All phthalate esters showed no estrogenic activity at concentrations between 4×10^{-7} mol/l and 1×10^{-4} mol/l. Further, judging from ALB induction, all phthalate esters showed no signs of toxicity to hepatocyte within the tested concentration range. Estrogenic potency relative to 100% E2 was estimated to be under 0.00001% for each compound (Table 1).

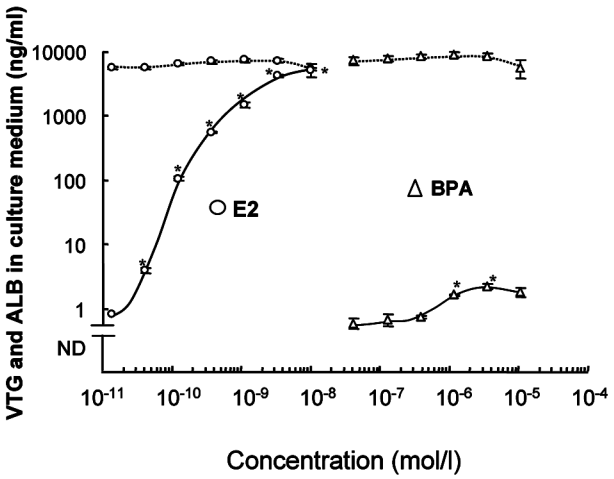

Fig. 2 Dose-response curves of BPA-dependent VTG (solid line) and ALB (dotted line) induction, compared with the results for E2. *: significant difference compared to the control ($p<0.05$). ND: <0.5 ng/ml. Each value indicates mean ± S.D. (n=3).

Table 1 Estrogenic potential of phthalate esters relative to E2, through induction of VTG synthesis

Compound	LOEC (M)[a]	Relative potency to E2 (%)[b]
17β-estradiol (E2)	4.1×10^{-11}	100
Bisphenol A (BPA)	1.1×10^{-6}	0.003
Dibutyl phthalate (DBP)	$>1.0\times10^{-4}$	< 0.00001
Butyl phthayl butyl glycolate (BPBG)	$>1.0\times10^{-4}$	< 0.00001
Benzyl butyl phthalate (BBP)	$>1.0\times10^{-4}$	< 0.00001
Benzyl benzoate (BB)	$>1.0\times10^{-4}$	< 0.00001

[a]LOEC is defined as lowest effective concentration among concentrations tested in this study.
[b]Relative potency was the ratio between concentrations of E2 and BPA required to induce 1 ng/ml VTG in culture medium.

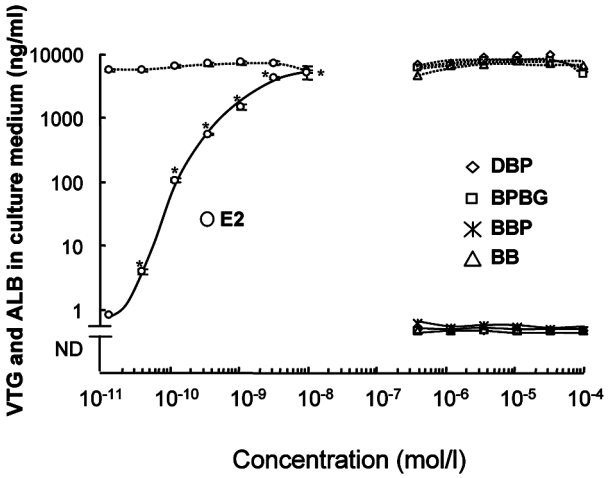

Fig. 3 Dose-response curves of phthalate ester-dependent VTG (solid line) and ALB (dotted line) induction, compared with the results for E2. *: significant difference compared to the control ($p<0.05$). ND: <0.5 ng/ml. Each value indicates mean ± S.D. ($n=3$).

DISCUSSION

Various technologies have been used to screen for EDCs — namely, MCF-7 cell proliferation assay, receptor binding assays, reporter gene expression assays using cultured cells and yeast cells, and a yeast two-hybrid assay[9–13]. For example, Hashimoto *et al.*[10] reported that BPA and BBP induced estrogenic activity at a concentration of 5×10^{-5} mol/l in a reporter gene assay (yeast two-hybrid system) and an estrogen/estrogen receptor (ER-α) competition binding assay (fluorescence polarization system). On the other hand, Harris *et al.*[12] reported that BBP and DBP showed weak estrogenic activity using a recombinant yeast screen for mitogenic effects on estrogen-responsive human breast cancer cells. BBP was estimated to be 1,000,000-fold less potent than E2. Likewise, Nishihara *et al.*[13] reported that BBP induced estrogenic activity at a concentration of 5×10^{-4} mol/l by yeast two-hybrid system, and was estimated to be 1,700,000-fold less potent than E2.

In the present study using *Xenopus* hepatocytes, the lowest effective concentration of E2 was 4×10^{-11} mol/l. Therefore, BBP was expected to induce VTG at a concentration around 1×10^{-5} mol/l. However, in parallel with DBP, BB, and BPBG, BBP showed no estrogenic activity at concentrations between 4×10^{-7} mol/l and 1×10^{-4} mol/l, while BPA showed weak activity at 1×10^{-6} mol/l. The results obtained in this study might mean that BBP was metabolized to non-estrogenic metabolites by *Xenopus* hepatocytes. In another report from NIES (National Institute of Environmental Studies, http://www.nies.go.jp/edc/estrogen/), when BBP was tested by *in vitro* yeast two-hybrid system in absence of rat liver S9 mix, it was estimated to be 130,000-fold less potent than E2; then, in the presence of rat liver S9 mix, BBP did not indicate any estrogenic activity. Taken together, the present result from *Xenopus* hepatocyte assay was in good agreement with the result obtained from yeast two-hybrid system in the presence of rat liver S9 mix.

On the other hand, Picard *et al.*[9] reported that while BBP — as a parent compound — stimulated MCF-7 proliferation in the E-Screen assay (at 10^{-6} mol/l up to 10^{-5} mol/l), none of its metabolites — which were identified in the culture medium — had estrogenic activities. They also reported that only 10% of the initial BBP remained in the culture medium after BBP was extensively metabolized by MCF-7 cells. By aligning our results with this report[9], our data might indicate that *Xenopus* hepatocytes have a high ability to metabolize xenobiotics, or that BBP has no estrogenic effects on *Xenopus laevis*. Concerning this assumption, it is necessary to know the binding activity of BBP to *Xenopus* estrogen receptor, although studies using competitive assays have found evidence that BBP does bind to the estrogen receptor in other species (rabbits, rats, and trouts)[14,18,19].

To evaluate the effects of chemicals using *in vitro* bioassays with cultured cells, it is important to provide evidence of practical viability through exposure testing. In this study, *Xenopus* hepatocytes were used. This was because *Xenopus laevis* is well established as an experimental animal and it is easy to prepare hepatocytes. Additionally, amphibians hold a peculiar ecological position because they experience both water and land environments in their life history. Indeed, the emergence of amphibians has generated new momentum for animal models. Their advantages as alternative experimental animals include developed four limbs, conversion of respiratory system from bronchial type to pulmonary type, and conversion of excretion from ammonia to urea/uric acid. Therefore, amphibians are considered to be a precious species as an environmental indicator among vertebrates.

In conclusion, *Xenopus* hepatocyte assay can directly estimate the estrogenic activity of phthalate esters in dental soft resins, whereby no estrogenic activities were shown in this study. Absence of estrogenic activity indicated that phthalate esters might be metabolically transformed into non-estrogenic substances in hepatocytes. To confirm the assumption, further study is needed to estimate the binding affinity of these phthalate esters to *Xenopus* estrogen receptor.

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