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In vivo estrogenic potential of 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, an active metabolite of bisphenol A, in uterus of ovariectomized rat

Katsuhiro Okuda, Masufumi Takiguchi, Shin'ichi Yoshihara*

Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hiro-koshingai, Kure, Hiroshima 737-0112, Japan

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ABSTRACT

4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), an active metabolite of bisphenol A (BPA), has more potent estrogenic activity than BPA *in vitro*, but its activity *in vivo* is not established. Here, we examined *in vivo* estrogenic activity of MBP by means of uterotrophic assay in ovariectomized (OVX) female rats. MBP exhibited dose-dependent estrogenic activity, as evaluated in terms of effects on uterus weight, uterine luminal epithelial cell height and myometrium thickness. The highest concentration of MBP (10 mg/kg/day) completely reversed the changes caused by OVX, and its activity was equivalent to that of 5 μg/kg/day 17β-estradiol (E2). We also investigated the effects of MBP on transcription of several estrogen-related genes. The changes of mRNA levels of estrogen receptors α and β, c-fos and insulin-like growth factor 1 in MBP-treated OVX rats were qualitatively similar to those in E2-treated rats. BPA did not show any significant effect on OVX rat in these conditions. This study is the first to demonstrate that MBP, an active metabolite of BPA, has potent *in vivo* estrogenic activity, being about 500-fold more potent than BPA in OVX rats.

1. Introduction

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is an endocrine-disrupting chemical (EDC) to which many people have potentially been exposed (Calafat et al., 2005; Kang et al., 2006; Völkel et al., 2008; Mielke and Gundert-Remy, 2009). It has been used as a plasticizer of polycarbonate and epoxy resin, which are extensively used in the manufacture of consumer goods and products, such as food containers, dental sealants, and protective coatings in metal cans for food and beverages (Brotons et al., 1995; Olea et al., 1996; Yamamoto and Yasuhara, 1999). Indeed, BPA has been detected in the serum of pregnant women, as well as fetal serum, placental tissue, amniotic fluid, follicular fluid and breast milk (Ikezuki et al., 2002; Schonfelder et al., 2002; Ye et al., 2006; Kuruto-Niwa et al., 2007). Moreover, significant amounts of BPA have been detected in the saliva of dental patients treated with fissure sealants (Olea et al., 1996). The results of *in vivo* studies on the estrogenicity of BPA are conflicting in terms of the effective dose. When pregnant CF-1 mice were fed with 2 ng/g or 20 ng/g body weight/day, the weight of male reproductive organs, such as prepuital gland and seminal vesicle, was affected in their offspring (Nagel et al., 1997; vom Saal et al., 1998), and Welshons et al. (2006) suggested that BPA also cause adverse effects in human at a concentration of real life exposure, namely low dose effect of BPA. However, other investigators could not reproduce these findings in male offsprings at the same dose (Ashby et al., 1999; Cagen et al., 1999).

We previously demonstrated that estrogenic activity of BPA in the yeast estrogen screening assay was increased after incubation with rat liver S9 fraction in the presence of an NADPH-generating system (Yoshihara et al., 2001). The active metabolite of BPA was identified as 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) by NMR and LC/MS/MS analysis (Yoshihara et al., 2004). MBP is produced by recombination of the one-electron oxidation product of carbon–phenyl bond cleavage of BPA, and its formation requires both microsomal and cytosolic fractions. Carbon–carbon bond cleavage of BPA was previously reported in MV-1, a Gram-negative aerobic bacterium isolated from enriched sludge taken from a wastewater treatment plant (Spivack et al., 1994), but was previously unknown as a mammalian metabolic mechanism.

It was subsequently shown that MBP has more potent estrogenic activity than BPA in several *in vitro* assays, by a factor of dozens to over one thousand times (Yoshihara et al., 2004). Moreover, MBP showed approximately 250- and 10,000-fold higher estrogenic activity than BPA in larvae and adult fish (*Oryzias latipes*), respectively, judging from an inducing ability of vitellogenin (Ishibashi et al., 2005; Yamaguchi et al., 2005). However, there has been no report on the activity of MBP in mammalians *in vivo*.

In this study, we evaluated the estrogenic activity of MBP in ovariectomized (OVX) female rats by means of uterotrophic assay. We also examined the effects of MBP on transcription of several

* Corresponding author. Tel.: +81 823 73 8945; fax: +81 823 73 8573.
E-mail address: s-yoshi@ps.hirokoku-u.ac.jp (S. Yoshihara).

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Table 1
Morphological and histological effects of estrogenic compounds on uterus in OVX rat.

	Dose (/kg/day)	Change of body weight (g)	Uterus weight (mg)	Uterine luminal epithelial cell height (μm)	Endometrium thickness (μm)	Myometrium thickness (μm)
Sham		1.5 ± 3.7*	534 ± 59*	7.0 ± 0.2	655 ± 94*	154 ± 10
Vehicle		10.3 ± 2.1	164 ± 18	4.9 ± 0.2	419 ± 26	132 ± 4
E_2	0.5 μg	5.3 ± 1.5	299 ± 8*	9.8 ± 1.2*	513 ± 10	147 ± 10
E_2	5 μg	1.3 ± 3.8*	503 ± 25*	9.2 ± 0.5*	481 ± 10	178 ± 5*
BPA	0.5 mg	7.8 ± 1.3	173 ± 3	2.2 ± 0.2	397 ± 23	107 ± 7
BPA	5 mg	9.8 ± 3.8	161 ± 10	2.3 ± 0.0	409 ± 6	111 ± 6
BPA	50 mg	5.8 ± 2.6	214 ± 12	3.1 ± 0.1	489 ± 41	134 ± 13
MBP	0.1 mg	7.3 ± 3.4	235 ± 14	3.7 ± 0.4	477 ± 42	137 ± 10
MBP	l mg	1.5 ± 1.3*	377 ± 44*	6.8 ± 0.8*	585 ± 77	180 ± 16*
MBP	10 mg	−8.3 ± 4.1*	530 ± 48*	8.7 ± 0.6*	607 ± 41	212 ± 18*

Each value represents the mean ± SEM (vehicle: $n = 3$; others: $n = 4$). The statistical significance of differences were assessed by using Dunnett's test.
* $P < 0.05$ as compared to the vehicle control group.

estrogen-related genes to examine the mechanism of its estrogenic activity.

2. Materials and methods

2.1. Chemicals

The sources of materials used were as follows: BPA was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 17β-Estradiol (E2) was purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Chemical synthesis of MBP was performed as previously reported (Yoshihara et al., 2004). MBP was obtained as a white powder giving a melting point at 130.5–132 °C. The ^{1}H NMR data (400 MHz, $CDCl_3$) of authentic MBP were as follows: δ 1.20 (s, 6H), 2.74 (s, 2H), 4.64 (s,1H), 4.71 (d, 1H, J = 2.0 Hz), 4.74 (s, 1H), 5.07 (d, 1H, J = 2.0 Hz), 6.67 (d, 2H, J = 8.8 Hz), 6.67 (d, 2H, J = 8.4 Hz), 7.09 (d, 2H, J = 8.8 Hz), 7.12 (d, 2H, J = 8.8 Hz). Other chemicals used were of the highest quality commercially available.

2.2. Animals

Female Wistar rats (11 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were housed under controlled temperature, lighting (12 h light:12 h darkness), and humidity (75 ± 5%) for at least 1 week prior to experiments. Standard pellet food and water were provided *ad libitum*. All animal studies were approved by the Institutional Review Board for Biomedical Research using Laboratory Animals at Hiroshima International University, and the animals were handled according to the institutional guidelines and regulations.

2.3. Uterotrophic assay in OVX rats

The assay has been performed in accordance with OECD TG 440. The animals were acclimatized to their environment for 1 week, then ovariectomized at 12 weeks of age and kept for 1 week before being used in experiments. Animals were treated subcutaneously with E2 at the concentration of 0.5 μg/kg/day or 5 μg/kg/day, or BPA (0.5 mg/kg/day, 5 mg/kg/day or 50 mg/kg/day), or MBP (0.1 mg/kg/day, 1 mg/kg/day or 10 mg/kg/day) dissolved in 5% ethanol in corn oil once daily for 5 days. All animals were sacrificed on the day after the last injection, and the uteri were removed. The uteri were trimmed of fat and any adhering nonuterine tissue, and blotted to remove excess fluid. Every uterus was weighed and fixed in 10% neutral buffered formalin. Portions of about 4 mm thickness were cut from above the junction with the uterine horn, and embedded in paraffin blocks. For histological examination, they were cut into multiple sections of 4 μm thickness, and stained with hematoxylin and eosin (HE). Corresponding areas of sequential sections were investigated by real-time PCR analysis as described below.

2.4. Sample preparation for real-time PCR analysis

Total RNA from uteri was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using a RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems, Inc., Carlsbad, CA), and reverse-transcription of extracted total RNA was performed with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Inc.), according to the manufacturers' protocols.

2.5. Real-time PCR

Real-time quantitative PCR analysis for estrogen receptor α (ERα) and β (ERβ), c-fos and insulin-like growth factor 1 (IGF-1) was performed using a StepOne real-time PCR System instrument and software (Applied Biosystems, Inc.).

Intron-spanning primers for the SYBR® Green assay were designed to meet specific criteria by using Primer Express software (Applied Biosystems, Inc.) and synthesized by Sigma–Aldrich Japan (Tokyo, Japan). The sequences of the PCR primer pairs that were used for each gene were as follows: ERα (forward: 5′-ATGATGAAAGGCGGGATACG-3′, reverse: 5′-TTCGGCCTTCCAAGTCATCT-3′, product length: 85 bp); ERβ (forward: 5′-ATGATCCTCCTCAACTCCAGTATGT-3′, reverse: 5′-CACCGCGTTCAGTAGGTGTGT-3′, product length: 93 bp); c-fos (forward: 5′-GGACAGCCTTTCCTACTACCATTC-3′, reverse: 5′-CGCAAAAGTCCTGTGTGTTGA-3′, product length: 79 bp); IGF-1 (forward: 5′-ACTGACATGCCCAAGACTCAGA-3′, reverse: 5′-TCTTGTTTCCTGCACTTCCTCTACT-3′, product length: 70 bp); β-actin (forward: 5′-AGATGACCCAGATCATGTTTGAGA-3′, reverse: 5′-ACCAGAGGCATACAGGGACAA-3′, product length: 86 bp).

PCR was performed with the Fast SYBR® Green Master Mix (Applied Biosystems, Inc.) using 2 μL of cDNA and 200 nmol/L of the primers in a 20 μL (final volume) of reaction mixture. Each of the 40 PCR cycles consisted of 3 s of denaturation at 95 °C, and hybridization of primers and polymerase reaction for 1 min at 60 °C.

The relative expression level of each gene of interest was computed with respect to the internal standard, β-actin, to normalize for variations in the quality of RNA and the amount of input cDNA. Therefore, the amount of target gene was divided by the endogenous reference amount to obtain a normalized value.

2.6. Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test to assess the significance of differences in mean values between the vehicle control group and drug treatment groups. Significance was accepted when the P value was less than 0.05.

3. Results

3.1. Uterotrophic assay in OVX rats

Rats were randomly divided into 10 groups. There was no significant difference of initial body weight among groups. The changes of body weight, calculated by subtraction of the body weight at 1 day before administration from that on the day after the last injection, are shown in Table 1. While the sham-operated rats and rats administered E2 (5 μg/kg/day) or MBP (1 mg/kg/day) showed weight gains of a just few grams, rats of the vehicle control group gained about 10 g. Some of the rats in the sham-operated, E2 (5 μg/kg/day) and MBP (10 mg/kg/day) groups showed decreased body weight, and the mean value of body weight change was negative in the MBP (10 mg/kg/day) group.

The uteri of vehicle control rats were significantly atrophied as compared with those of sham-operated rats, because of the depletion of endogenous estrogen due to OVX. The atrophy was dose-dependently reversed by E2 (0.5 and 5 μg/kg/day) and MBP (1 and 10 mg/kg/day). The highest dose of both compounds completely restored the uterus weight. BPA had no effect on the uterus weight at any dose.

Histologically, the uterine luminal epithelial cell height of vehicle control rats became lower than that of sham-operated rats. This change was fully reversed by E2 (0.5 μg/kg/day or 5 μg/kg/day) and MBP (1 mg/kg/day or 10 mg/kg/day). In addition, a significant decrease of endometrium thickness was observed in vehicle control rats, but this was not significantly reversed by any of the test compounds. Myometrium thickness, which was slightly, though