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Ipso substitution of bisphenol A catalyzed by microsomal cytochrome P450 and enhancement of estrogenic activity

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ABSTRACT

Bisphenol A (BPA), an industrial chemical with estrogenic activity, was investigated as a substrate for the *ipso*-metabolism catalyzed by microsomal cytochrome P450 (P450). BPA was expected to be transformed to a quinol *via* an *ipso*-addition reaction; however, hydroquinone (HQ) was detected as a metabolite *via* an *ipso*-substitution reaction. Isopropenylphenol (IPP) and hydroxycumyl alcohol (HCA) were also produced as eliminated metabolites by C-C bond scission *via ipso*-substitution. Incorporation of the ^{18}O atom to HCA from $H_2{}^{18}O$ suggested the presence of a carbocation intermediate. Bulkiness of *p*-substituted group of BPA and/or stability of the eliminated carbocation intermediate may cause *ipso*-substitution of BPA. CYP3A4 and CYP3A5 showed higher activity for *ipso*-substitution. CYP2D6*1 also showed the activity; however, the other 9 isozymes did not. IPP showed ER-binding activity in the same degree of BPA. Furthermore, the ER-binding activity of HCA was about a hundred times greater than that of BPA. These results suggested that this new metabolic pathway contributes to the activation of the estrogenic activity of BPA.

1. Introduction

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is one of the most widely used industrial chemicals for the production of resins and plastics. This compound is considered to be an endocrine disrupter (Krishman et al., 1993). BPA is widely detected from human urine samples (Calafat et al., 2005); therefore, the clarification of its metabolism has value.

Cytochrome P450 (P450) is an enzyme that catalyzes the oxidation of a wide variety of xenobiotic chemicals, including drugs (Ortiz de Montellano, 2005). BPA is known to be metabolized to 2,2-bis(4-hydroxyphenyl)propanol, *o*-hydroxybisphenol, or bisphenol-*o*-quinone as an oxidative product by P450, and the glucuronide is a major conjugated metabolite (Knaak and Sullivan, 1966; Jaeg et al., 2004). Yoshihara et al. (2001) reported that 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) was formed from BPA by an S9 fraction and its estrogenic activity was higher than that of BPA (Okuda et al., 2010).

We have previously demonstrated that the *ipso*-position metabolism reaction of *para*-substituted phenols is catalyzed by P450 (Fig. 1). In other words, when a substituent is a member of the alkyl group, a quinol metabolite is formed through an *ipso*-addition reaction. On the other hand, when a substituent is a carboxyl, acetyl, or hydroxymethyl group, a hydroquinone (HQ) is formed by an *ipso*-substitution reaction (Ohe et al., 1994, 1995, 1997). Estrone and 17β-estradiol, each of which contains a *para*-alkylphenol moiety, are also metabolized through *ipso*-addition to the corresponding quinols by P450 (Ohe et al., 2000). Nonylphenol, an endocrine disrupter, is also metabolized to the corresponding quinol, which has no estrogenic activity (Tezuka et al., 2007). In the case of nonylphenol, the *ipso*-position metabolism by P450 led to metabolic inactivation.

BPA also contains a *para*-alkylphenol moiety; therefore, it is expected to be metabolized by an *ipso*-addition reaction. In this study, the new metabolic pathway of BPA catalyzed by rat liver microsomes and human P450 (CYP) is investigated. The estrogenic activity of novel metabolites is also examined.

2. Materials and methods

2.1. Chemicals

The sources of the materials used were as follows: BPA; hydroquinone; and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase (G-6-P DHase) were obtained from Roche Diagnostics (Basel, Switzerland). $NADP^+$ was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *p*-Acetoxybenzophenone and a methylmagnesium iodide solution were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Methyltriphenylphosphonium bromide was obtained from Sigma Aldrich Co. (St. Louis, MO). Water-^{18}O (≥98 atom% ^{18}O) was obtained from Taiyo Nippon Sanso Co. (Tokyo, Japan). *o*-Hydroxybisphenol A (o-

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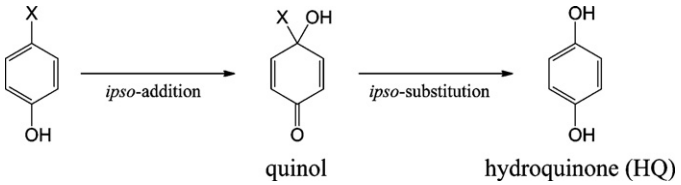

Fig. 1. *ipso*-Position metabolism catalyzed by cytochrome P450. When the *p*-substituted group is an alkyl group, the corresponding quinol is stably obtained. When the *p*-substituted group is halogen, hydroxymethyl, acetyl, nitro, cyano, or carboxyl, the *p*-substituted group is eliminated to form HQ *via* a quinol intermediate.

OH-BPA) was synthesized as described method (Atkinson and Roy, 1995). Other chemicals used were of the highest quality commercially available.

2.2. Preparation of rat liver microsomes

Wistar/ST rats (male, 6 weeks old, 200–220 g each) were intraperitoneally injected with phenobarbital (60 mg/kg in saline) for 3 days and sacrificed 24 h after the last injection. The liver microsomes were prepared in accordance with previously described procedures (Masumoto et al., 1989).

The microsomal protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard, and the P450 content was measured as described by Omura and Sato (1964).

2.3. Rat liver microsomal incubation

The incubation mixture containing rat liver microsomes (4 mg protein; 8–10 nmol P450), a substrate (1 mM), KCl (60 mM), $MgCl_2$ (4 mM), G-6-P (4 mM), and G-6-P DHase (5 units) in 2.5 ml of a 0.1 M sodium phosphate buffer (pH 7.4) was preincubated for 3 min at 37 °C. The reaction was initiated by adding $NADP^+$ (final 0.4 mM). In the case of the $H_2{}^{18}O$-enriched reaction, the final $H_2{}^{18}O$ content in the reaction mixture was adjusted to 50%. After being incubated for 30 min at 37 °C, the mixture was treated with 2 mL of ice-cold ethyl acetate to stop the reaction and extract the products, and the organic phase was separated. The organic phase was dried over anhydrous Na_2SO_4 and concentrated by nitrogen flushing. The products formed were trimethylsilylated with BSTFA and pyridine. After the removal of the excess BSTFA by nitrogen flushing, the residue was dissolved in a small amount of acetone and analyzed by GC-MS (JEOL AUTOMASS SUN200, capillary column HP-1 0.32 mm × 30 m; J & W Scientific). The mobile phase was He and the flow rate was 2.0 mL/min. The injection temperature was 260 °C. The initial column temperature was 90 °C for 3 min; it was then raised at intervals of 10 °C/min to 250 °C, followed by an isothermal hold at this temperature. The peaks were detected by EI mode.

2.4. Human liver microsomes and cytochrome P450 (CYP)

Pooled human liver microsomes were obtained from Gentest Co. (Woburn, MA). Pool is comprised of 22 specimens (13 males and 9 females). Recombinant CYP1A1, 1A2, 2B6, 2C9, 2C19, 2D6*1 (CYP2D6-Val_{374}), 2D6*10 (P34S and S486T), 2E1, 3A4, 3A5, 3A7, and 4A11 expressed in the microsomes of insect cells (BTI-TN-5B1-4) infected with a baculovirus containing CYP and NADPH-CYP reductase cDNA inserts and control microsomes (expressed with NADPH-CYP reductase and cytochrome b_5) were also obtained from Gentest.

2.5. Incubation with human liver microsomes or CYP isozymes

Incubation with human liver microsomes (4 mg protein; 1.44 nmol P450) was performed on the same condition with rat liver microsomes as described above. Incubation with cDNA expressed-CYP isozymes was performed similarly, except that the incubation was carried out in a final volume of 1 mL and the incubation mixture contained 0.1 μM CYP isozymes, a 0.1 mM substrate, 60 mM KCl, 4 mM $MgCl_2$, 4 mM G-6-P, 5 units of G-6-P DHase, and 0.4 mM $NADP^+$.

2.6. Estrogen receptor binding reporter gene assay

For the estrogen-responsive element (ERE)-luciferase reporter gene assay using MCF-7 cells, the culture medium was changed to phenol red-free MEM (Sigma Chemical Co.) containing penicillin, streptomycin, and dextran-charcoal-treated fetal bovine serum for 2–3 days. Transient transfections to MCF-7 cells were performed using Transfast (Promega Co., Madison, WI), in accordance with the manufacturer's protocol. The transfections were conducted in 12-well plates at 1×10^5 cells/well with 1.9 μg of p(ERE)3-SV40-Luc and 0.1 μg of pRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after the addition of the sample (final concentration, 10^{-5} to 10^{-10} M) dissolved in 10 μL of ethanol, the assay was performed with a Dual Luciferase assay kit (Promega Co.) in accordance with the manufacturer's protocol.

2.7. Synthesis of 4-hydroxycumyl alcohol (HCA)

In a round-bottom flask were placed *p*-acetoxyacetophenone (0.89 g) and diethyl ether (20 mL) under nitrogen. A methylmagnesium iodide solution (16.6 mL) was added dropwise at room temperature, and the resulting mixture was stirred for 5 h. After addition of saturated aqueous ammonium chloride (100 mL), the reaction mixture was extracted with ethyl acetate. The organic phase was washed with brine and dried over anhydrous magnesium sulfate. The crude product (0.60 g) was obtained after the solvent was evaporated *in vacuo*. Finally, 0.21 g of 4-hydroxycumyl alcohol was obtained by silica gel chromatography eluted with *n*-hexane/ethyl acetate. ^{1}H NMR (500 MHz, CD_3OD): δ 1.48 (*s*, 6H), 6.71 (*d*, 2H, J = 8.5 Hz), 7.28 (*d*, 2H, J = 8.5 Hz); MS (EI) as TMS-derivative *m*/*z*: 296 [M^+], 281 [M^+-CH_3], 207 [M^+-OTMS].

2.8. Synthesis of 4-isopropenylphenol (IPP)

Methyltriphenylphosphonium bromide (5.4 g) and diethyl ether (20 mL) were placed in a round-bottom flask under nitrogen. A solution of 10% potassium *t*-butoxide in *t*-butanol (17 mL) was added dropwise at room temperature, and the resulting mixture was stirred for 1 h. After the addition of *p*-hydroxyacetophenone (0.68 mg) in diethyl ether (10 mL), the reaction mixture was stirred for 12 h at 50 °C. The reaction mixture was added with saturated aqueous ammonium chloride and extracted with ethyl acetate. The organic phase was washed with brine and dried over anhydrous magnesium sulfate. The crude product (2.9 g) was obtained after the solvent was evaporated *in vacuo*. Finally, 0.25 g of 4-isopropenylphenol was obtained by silica gel chromatography eluted with *n*-hexane/ethyl acetate. ^{1}H NMR (500 MHz, CD_3OD): δ 2.08 (*s*, 3H), 4.90 (*s*, 1H), 6.72 (*d*, 2H, J = 8.5 Hz), 7.31 (*d*, 2H, J = 8.5 Hz); MS (EI) as TMS-derivative *m*/*z*: 206 [M^+], 191 [M^+-CH_3].

3. Results

3.1. Metabolism of BPA by rat or human liver microsomes

BPA was incubated with phenobarbital-treated rat liver microsomes or pooled human liver microsomes. We expected that a quinol of BPA would be produced as a metabolite of *ipso*-addition reaction because BPA contains a *para*-alkyl group. The metabolite extracts were analyzed by GC-MS after trimethylsilylation, but no *ipso*-addition product was detected. On the other hand, HQ was detected as a metabolite *via* an *ipso*-substitution reaction (Table 1). Trimethylsilylated HQ was identified on the basis of retention time and the *m*/*z* peak ratio of 254/239/223/112 in the GC-SIM mode, compared with the authentic standard. The novel metabolites IPP and HCA were also detected as a part of BPA after dissociation of HQ (Table 1). Trimethylsilylated IPP and HCA were identified on the basis of the retention time and the *m*/*z* peak ratio of 206/191/151/115 and 296/281/207/191 in the GC-SIM mode, compared with the synthetic standard, respectively. *o*-Hydroxybisphenol A (*o*-OH-BPA) was also detected as a known metabolite by P450. Trimethylsilylated *o*-OH-BPA was identified on the basis of the retention time and the *m*/*z* peak ratio of 460/445/207 in the GC-SIM mode, compared with the authentic standard. All metabolites were successfully extracted by the method described in Section 2.3.

3.2. Incorporation of ^{18}O from $H_2{}^{18}O$ into metabolites

When BPA was incubated with rat liver microsomes in an $H_2{}^{18}O$-enriched reaction buffer, incorporation of ^{18}O into metabolites was estimated from the ratio of the peak areas of [M^++2] and [M^+] by GC-SIM. The ratio of [M^++2]:[M^+] was 43.8:56.2, therefore the incorporation ratio of ^{18}O from $H_2{}^{18}O$ into HCA was calculated as 87.6%. On the other hand the ratio into HQ was 0.1%.

3.3. Metabolism of BPA by isozymes of human cytochrome P450

BPA was incubated with human cytochrome P450 (CYP) isozymes, and the amounts of formed HQ and HCA were determined (Table 2). The amounts of IPP could not be determined because of the interference by a large peak close to the peak of IPP.