Metabolism and Interaction of Bisphenol A in Human Hepatic Cytochrome P450 and Steroidogenic CYP17

Toshiro Niwa, Megumi Fujimoto, Kae Kishimoto, Yoshiyasu Yabusaki, Fumihide Ishibashi, and Masanao Katagiri

Division of Natural Science, Osaka Kyoiku University; and Biotechnology Laboratory, Sumitomo Chemical Co., Ltd.

The metabolism of bisphenol A (BPA) was determined for 11 forms of human hepatic cytochromes P450 (CYPs) expressed in the yeast Saccharomyces cerevisiae and for human steroidogenic CYP17 expressed in Escherichia coli. Additionally, the effect of BPA on the progesterone 17α-hydroxylase activity of CYP17 was investigated. CYP2C18 catalyzed BPA metabolism most efficiently, followed by CYP2C19 and CYP2C9. CYP2C9 and CYP2C18 exhibited the highest affinity (Km=3.9 μM) for BPA metabolism. The Vmax of CYP2C18 (8.10 nmol·min⁻¹·nmol CYP⁻¹) was 5 times higher than that of CYP2C9. Although the Vmax of CYP2C19 was 1.5 times higher than that of CYP2C18, the affinity of CYP2C19 was 12 times lower than that of CYP2C9 and CYP2C18. Therefore the intrinsic clearance (Vmax/Km) of CYP2C18 was more than 5 times higher than that of CYP2C9 and CYP2C19. On the other hand, BPA exhibited a competitive-type inhibition of the progesterone 17α-hydroxylase activity of CYP17 with a Ki value of 71 μM, whereas no metabolism of BPA by CYP17 was detected. These results suggest that BPA is mainly metabolized by the CYP2C subfamily in human liver, and that BPA inhibits human steroidogenic CYP17 activities.

Key words: bisphenol A; human hepatic cytochrome P450; human CYP17; progesterone 17α-hydroxylase

Bisphenol A (4,4'-isopropylidenediphenol, BPA) is widely used in the chemical industry in the manufacturing of epoxy, polycarbonate, and polyester-styrene resins, and trace levels of BPA leach from polycarbonate plasticware and resins used for food packaging materials. This compound is suspected to be an endocrine disruptor. Additionally, it has been reported that exposure of male rats and mice to BPA may be associated with increased incidence of cancers of the hematopoietic system and that high doses of BPA cause reproductive toxicity and affect cellular development in these species. However, the effect of BPA in vivo and its mechanism of action are still unclear.

Cytochrome P450 (CYP) comprises a superfamily of enzymes that catalyze the oxidation of a wide variety of xenobiotic chemicals including drugs, carcinogens, and steroids including sex hormones. In the rat, BPA is metabolized to DNA-reactive bisphenol-α-quinone through 5-hydroxybisphenol and bisphenol semiquinone, and the formation of the DNA adducts in a microsomal activation system is markedly decreased by CYP inhibitors, suggesting that CYPs are closely associated with the metabolism and toxicity of BPA in rats. However, there are few reports on BPA metabolism in humans and on the human CYP(s) that metabolizes BPA.

Recently, we reported that BPA inhibits human hepatic CYP-mediated drug-metabolizing activities including aminopyrine N-demethylation, especially by CYP2C8 and CYP2C19. Additionally, Hanioka et al. reported that the administration of BPA to male rats decreased the catalytic activities and protein levels of male-specific CYP isofoms (such as CYP2C11 and CYP3A2) in rat liver microsomes, and that BPA inhibited the drug-metabolizing activities of rat hepatic CYP1A2, CYP2A2, CYP2B2, CYP2C11, CYP2D1, CYP2E1, and CYP3A2.

CYP17 is found in the endoplasmic reticulum of the adrenal cortex and gonads, and mediates both 17α-hydroxylase and 17,20-lyase reactions of pregnenolone and progesterone, and is thus involved in the biosynthesis of glucocorticoids and sex hormones. Therefore endocrine disrupters (sex hormone-like compounds) may affect the activity of CYP17. Recently, we reported that the aminopyrine N-demethylase activity of CYP17 was comparable with that of CYP3A4, a dominant CYP in human liver.

This paper describes an in vitro investigation of BPA metabolism by human hepatic CYPs and steroidogenic CYP17 based on the disappearance rate of parent compounds from an incubation mixture and the inhibitory effect of BPA on the progesterone 17α-hydroxylase activity of CYP17.

MATERIALS AND METHODS

Materials Bisphenol A was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Progesterone, 17α-hydroxyprogesterone, and estrone were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human NADPH-cytochrome P450 reductase (fp2) and hydrocortisone acetate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of the highest purity commercially available.

Expression of Human CYPs and the Preparation of Microsomes and Membrane Fraction The expression of human hepatic CYPs in recombinant Saccharomyces cerevisiae and preparation of microsomal fractions from the yeast cells were carried out according to the methods described previously. The expression of human adrenal CYP17 in Escherichia coli and preparation of membrane fractions from the cells were carried out by the methods of Imai et al. and Katagiri et al. The contents of CYPs were determined as described by Omura and Sato.

Assay of Disappearance of BPA The identification of CYP isofom(s) has generally been performed by measuring the metabolite(s) production rate with a specific pathway of metabolism. However, because the metabolic process of toxic
compounds and new drugs in the early stage of drug development is not completely elucidated, the disappearance rate of a compound from incubation medium may be useful for the identification of the responsible CYP isoform(s).\textsuperscript{18,19}

For the standard assay with hepatic CYPs, the incubation mixture consisting of yeast microsomes (20—50 pmol of CYP), BPA 1—500 \( \mu \text{M} \), NADPH 1 mM, and Tris–HCl buffer 100 mM (pH 7.5) in a final volume of 0.5 ml. After 2-min preincubation at 37 °C, the reaction was started by adding NADPH. Incubation was carried out at 37 °C for 10 min, and the reaction was terminated by the addition of ethyl acetate 3 ml, and 50 \( \mu \text{l} \) of estrone 40 or 1000 (for kinetic study) \( \mu \text{M} \) was added as an internal standard. After ethyl acetate extraction, the organic phase (2.5 ml) was evaporated under nitrogen, the residue was dissolved in 200 or 1000 (for kinetic study) \( \mu \text{l} \) of the high-performance liquid chromatography (HPLC) mobile phase and analyzed by HPLC. The HPLC system consisted of an L-7100 pump, an L-7400 UV-detector set at 230 nm, and a D-7500 integrator (Hitachi). A column (4.6×250 mm) packed with Cosmosil 5C18-AR-II (Nacalai Tesque) was used. The mobile phase consisted of phosphoric acid 7.5 \( \mu \text{M} \)/acetonitrile (60 : 40), and the flow rate was 1 ml/min. The calibration curve was linear for BPA concentrations ranging from 0.1 to 100 \( \mu \text{M} \), and the coefficients of variation were less than 6.6%.

For the assay with CYP17, the membrane fraction containing CYP17 50 pmol and fp 2 150 pmol were preincubated at 37 °C for 3 min, and the mixture was brought to 450 \( \mu \text{l} \) by addition of phosphate buffer 100 mM (pH 7.4) and BPA 1 \( \mu \text{M} \). The reaction was started by adding 50 \( \mu \text{l} \) of NADPH 10 mM. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 3 ml of ethyl acetate, and 50 \( \mu \text{l} \) of estrone 40 \( \mu \text{M} \) was added as an internal standard. BP A in the incubation mixture was determined as described above.

Assay of Progesterone 17\( \alpha \)-Hydroxylase Activity Progesterone 17\( \alpha \)-hydroxylase activity was determined by the methods described previously\textsuperscript{13,16} with minor modification. Briefly, the membrane fraction containing CYP17 30 pmol and fp 90 pmol were preincubated at 37 °C for 3 min, and the mixture was brought to 450 \( \mu \text{l} \) by addition of phosphate buffer 100 mM (pH 7.4), magnesium acetate 10 mM, and progesterone 1—50 \( \mu \text{M} \). The reaction was started by adding 50 \( \mu \text{l} \) of NADPH 10 mM. Incubation was carried out at 37 °C for 20 min, and the reaction was terminated by the addition of 2 ml of ethyl acetate, and 50 \( \mu \text{l} \) of hydrocortisone acetate 100 \( \mu \text{M} \) was added as an internal standard. After ethyl acetate extraction, the organic phase (1.5 ml) was evaporated under nitrogen, and the residue was dissolved in 200 \( \mu \text{l} \) of the HPLC mobile phase and analyzed by HPLC. The same HPLC system and column as described above were used, except that the UV-detector was set at 240 nm. The mobile phase consisted of methanol/acetonitrile/water (55 : 10 : 35), and the flow rate was 0.75 ml/min.

Kinetic Analysis \( V_{\text{max}} \) and \( K_m \) values for the disappearance of BPA were determined by fitting to Michaelis–Menten kinetics with Lineweaver–Burk plots, and the inhibition constants (\( K_i \) values) were estimated from the plot of \( K_i/V_{\text{max}} \) and \( K_{\text{m, app}}/V_{\text{max}} \) as a function of increasing inhibitor concentrations.

RESULTS

BPA Metabolism by Human CYPs The disappearance of BPA at a substrate concentration of 1 \( \mu \text{M} \) was investigated in 11 forms of human hepatic CYPs (Fig. 1). CYP2C18 most efficiently catalyzed BPA metabolism (1.65 nmol·min\(^{-1} \cdot \mu \text{mol CYP}\)), and CYP2C19 and CYP2C9 exhibited 14—20% of the activity of CYP2C18. CYP1A1, 2B6, and 2C8 catalyzed BPA metabolism, but at a much lower rate than
CYP2C18 (4—10%), and no activity of CYP1A2, 2A6, 2D6, 2E1, and 3A4 was detected. Metabolism of BPA by CYP17 was not observed (<0.05 nmol·min⁻¹·nmol CYP⁻¹, data not shown).

Kinetics of BPA Metabolism The kinetics of BPA metabolism by CYP2C9, CYP2C18, and CYP2C19 were further studied by fitting to the Michaelis–Menten kinetics with Lineweaver–Burk plots (Fig. 2). CYP2C18 exhibited the highest affinity with respect to BPA metabolism ($K_m = 3.9 \mu M$), and high ability of the metabolism ($V_{max} = 8.10$ nmol·min⁻¹·nmol CYP⁻¹). CYP2C9 also had the highest affinity ($K_m = 3.9 \mu M$), whereas its $V_{max} (1.62 \text{ nmol·min}^{-1} \cdot \text{nmol CYP}^{-1})$ was 20% of that of CYP2C18. Although the $V_{max}$ of CYP2C19 was 1.5 times higher than that for CYP2C18, the $K_m$ of CYP2C19 was 12 times higher than that of CYP2C9 and CYP2C18. Therefore the intrinsic clearance ($V_{max}/K_m$) for CYP2C18 (2.07 nmol·min⁻¹·nmol CYP⁻¹) was more than 5 times greater than that of other CYPs.

Inhibitory Effect of BPA on Progesterone 17α-Hydroxylation by CYP17 The inhibitory effect of BPA on progesterone 17α-hydroxylation by CYP17 was investigated at concentrations of 100 and 300 $\mu M$ (Fig. 3). In the absence of BPA, $K_m$ and $V_{max}$ values for progesterone 17α-hydroxylation by CYP17 were 2.26 $\mu M$ and 2.03 nmol·min⁻¹·nmol CYP⁻¹, respectively. In the presence of increasing BPA concentrations, a concentration-dependent inhibition of progesterone 17α-hydroxylation was characterized by an increasing $K_m$ value, whereas $V_{max}$ remained constant. This is consistent with a competitive-type inhibition of progesterone 17α-hydroxylation by BPA, and the $K_i$ value for BPA was calculated to be 71 $\mu M$.

DISCUSSION

The metabolism of BPA was investigated in 11 forms of human hepatic CYPs and for human steroidogenic CYP17. CYP2C18 most efficiently catalyzed BPA metabolism, followed by CYP2C19 and CYP2C9. CYP1A1, 2B6, and 2C8 also catalyzed BPA metabolism, but the activities of CYP1A2, 2A6, 2D6, 2E1, and 3A4 were negligible. Additionally, among CYP2C9, CYP2C18, and CYP2C19, CYP2C18 exhibited the highest affinity for BPA metabolic activity, and a high ability for metabolism, CYP2C9 also showed the same affinity, whereas its $V_{max}$ was 20% of that of CYP2C18. Although the $V_{max}$ of CYP2C19 was 1.5 times higher than that of CYP2C18, the $K_m$ value for CYP2C19 was 12 times higher than that for CYP2C9 and CYP2C18; the intrinsic clearance ($V_{max}/K_m$) of CYP2C18 was more than five-fold that of CYP2C9 and CYP2C19. However, Shimada and colleagues reported that the relative CYP3A4/5, 2C8/9/18/19, 1A2, 2E1, and 2A6 contents in 60 human liver samples were 29%, 18%, 13%, 7%, and 4%, respectively, and that CYP2C9 and CYP2C19 levels were 17—20% and 0.8—1.4%, respectively, of total CYP in the liver in Japanese and Caucasian populations.

Additionally, CYP2C18 appears to be expressed at a very low level in the human liver. Based on the respective proportions of these CYP isoforms and their specific turnover for BPA elimination determined on recombinant CYPs, it is possible to speculate that CYP2C9 is mainly involved in BPA metabolism. Further detailed studies using human liver microsomes are required. On the other hand, no metabolism of BPA by adrenal steroidogenic CYP17 was observed.

The CYP2C subfamily in the human liver comprises four members: CYP2C8, CYP2C9, CYP2C18, and CYP2C19. CYP2C9 and CYP2C19 are 92% identical in their amino acid sequences, but they have very distinctive substrate specificities. In this study, BPA was metabolized by CYP2C9 and CYP2C19 as well as by CYP2C18. However, both $K_m$ and $V_{max}$ values for CYP2C19 were 7.5 to 12.4 times higher than those for CYP2C9, indicating that CYP2C9 and CYP2C19 differ in their affinity for and ability in BPA metabolism.

It has been reported that BPA is metabolized to DNA-reactive bisphenol-o-quinone through 5-hydroxybisphenol and bisphenol semiquinone in rats, and that the formation of DNA adducts in a microsomal activation system is markedly decreased by CYP inhibitors, suggesting that CYPs are closely associated with the metabolism and toxicity of BPA. From our results, it is possible to speculate that in humans, the biotransformation from BPA to hydroxylated BPA is catalyzed by the hepatic CYP2C subfamily.

We have reported the effect of BPA on human hepatic CYP activities using aminopyrine as a substrate, because most CYPs show aminopyrine N-demethylase activity; the activities of CYPs except for CYP2E1 were inhibited by BPA. Additionally, BPA noncompetitively inhibited the aminopyrine N-demethylase activity by CYP2C8, and exhibited a mixed-type inhibition for S-mephenytoin 4-hydroxylation by CYP2C19. However, the inhibitory effect of BPA on steroidogenic CYPs including CYP17 is not known. Therefore we investigated the effect of BPA on progesterone 17α-hydroxylation by human CYP17. In the absence of BPA, $K_m$ and $V_{max}$ values for progesterone 17α-hydroxylation by CYP17 were 2.26 $\mu M$ and 2.03 nmol·min⁻¹·nmol CYP⁻¹, respectively (Fig. 3); the $K_m$ and $V_{max}$ values were comparable with those reported previously. In this condition, BPA competitively inhibited progesterone 17α-hydroxylation by human CYP17; the inhibition constant ($K_i$) was 71 $\mu M$. Biles et al. reported that the levels of BPA from epoxy-coated can surfaces containing infant formula concentrates ranged from 0.1 to 13 ppb (57 nM), and Brotons et al. extracted BPA from the liquid of canned food ranging from 0 to 33 $\mu$g per can. However, there are few reports on BPA con-
centrations in plasma or adrenal glands after dosing and protein binding. To evaluate further the potential hazards posed by BPA to humans, more information is needed on exposure and pharmacokinetics.

In summary, we showed that BPA was predominantly metabolized by the CYP2C subfamily (CYP2C9, CYP2C18, and CYP2C19) in human liver, and that BPA inhibited human steroidogenic CYP17 activities.

REFERENCES AND NOTES