Metabolic Activation of Bisphenol A by Rat Liver S9 Fraction
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Bisphenol A (BPA) is a well-known endocrine-disrupting chemical found in the environment. To assess the metabolic modulation of estrogenic activity of BPA after ingestion, we investigated whether the incubation of BPA with rat liver S9 fraction results in metabolic activation or inactivation of estrogenic activity using a recombinant yeast expressing human estrogen receptor and MCF-7 transfected firefly luciferase plasmid for a reporter assay. When 0.1 mM BPA was incubated with rat liver S9 for 1 h, the estrogenic activity was increased about two to five times compared with that of the control, in which the S9 was inactivated prior to incubation. This metabolic activation was inhibited by SKF 525-A, an inhibitor of cytochrome P450. With increasing incubation time, the estrogenic activity increased time-dependently. Interestingly, however, the metabolic activation did not proceed with either microsomes or cytosol alone and was restored by a recombination of both fractions. The active metabolite was eluted at later retention time than that of BPA on HPLC with a reversed-phase column. Bisphenol B and methoxychlor were also activated by incubation with rat liver S9, whereas 4-tert-octylphenol and 4-nonylphenol, as well as 17β-estradiol, were metabolically inactivated. The present results clearly indicate that BPA is metabolically activated in terms of estrogenicity under the conditions existing only with combined rat liver microsomes and cytosol.

Key Words: bisphenol A; 17β-estradiol; estrogenic activity; recombinant yeast assay; MCF-7; rat liver S9; metabolic activation.

In recent years there has been increasing public concern that chemicals in the environment may affect the endocrine function of humans and wildlife (Colborn, 1995). It is already known that a wide variety of chemicals have estrogenic activity. These substances include phytoestrogens such as daidzein and genistein and manmade chemicals such as pesticides, plasticizers, and breakdown products of surfactants. Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane, is a monomer of epoxy and polycarbonate plastics widely used in consumer products. BPA was also identified as the estrogenic contaminant released from the coated inner plastic lining of food cans (Brotons et al., 1995) and from dental sealants and composites (Olea et al., 1996). Accordingly, BPA is regarded as one of the endocrine-disrupting chemicals (EDCs) most likely to be ingested in our daily life. Judging from the high LD50 values of 4.24 g/kg for rats and 2.5 g/kg for mice (AIHA, 1967; NIOSH, 1978), the acute toxicity of BPA appears not to be high. However, there are several reports suggesting hazardous effects of BPA (Hardin et al., 1981; Maguire, 1988; Reel et al., 1985), including DNA adduct formation (Atkinson and Roy, 1995). Estrogenicity of BPA was found incidentally by Feldman et al. (1984) as a result of autoclaving polycarbonate flasks. Subsequently, they demonstrated that BPA promotes cell proliferation and progesterone receptor synthesis in estrogen-responsive MCF-7 human breast cancer cells (Krishnan et al., 1993). On the other hand, the results of in vivo studies on the estrogenicity of BPA are very controversial in terms of the dose affected. The low-dose (2 or 20 μg/kg/day) effects of BPA on male reproductive organs such as prostate glands, preputial glands, and epididymides in CF-1 mice exposed during prenatal development were reported by Nagel et al. (1997) and vom Saal et al. (1998). However, other investigators have not observed such effects of BPA administered to pregnant CF-1 mice even at the same dose (Ashby et al., 1999; Cagen et al., 1999).

Recently, a novel metabolic pathway of BPA in MV1, a Gram-negative aerobic bacterium isolated from enriched sludge taken from a wastewater treatment plant, was demonstrated by Spivack et al. (1994). The major route of metabolism (>80%) in this bacterial strain was oxidative cleavage of an intermediary metabolite, 4,4′-dihydroxy-α-methylstilbene, to 4-hydroxybenzaldehyde and 4-hydroxyacetophenone. It is noteworthy that the chemical structure of 4,4′-dihydroxy-α-methylstilbene is similar to that of diethylstilbestrol, a potent synthetic estrogen. Knaak and Sullivan (1966) first reported the metabolic fate of BPA in rats, showing that the major metabolite in urine was the glucuronide of BPA; considerable amounts of free BPA and hydroxylated BPA were found in feces. Recently, two groups of investigators reconfirmed that the predominant metabolite in urine of rats is BPA monoglucuronide (Pottenger et al., 2000; Snyder et al., 2000). On the contrary, Miyakoda et al. (1999; 2000) have shown that BPA, not the glucuronide, was detected in fetuses after oral administration of BPA to the pregnant rats. This observation is very suggestive. The fetus must be a primary target of BPA; nevertheless, fetal liver may lack the ability to inactivate BPA to the glucuronide, which is essentially inactive as an estrogen (Snyder et al., 2000). In this connection, Steinmetz et al. (1997) found that although the potency of BPA in stimulating
prolactin gene expression and release in vitro was 1000- to 5000-fold lower than that of 17β-estradiol (E2). BPA showed similar potency to E2 in stimulating prolactin release in vivo in estrogen-sensitive Fisher 344 rats. The discrepancy between the estrogenic potency of BPA in vitro and in vivo may suggest the formation of active metabolite(s) in vivo.

Needless to say, in any assessment of the in vivo impact of EDCs, the metabolic modulation of estrogenic activity must be considered. Therefore, we investigated how the metabolism of EDCs (especially BPA) by rat liver S9 fraction affects their estrogenic activity, other than the conjugation reaction, using recombinant yeast and MCF-7 reporter assays.

**MATERIALS AND METHODS**

*Chemicals.* The sources of materials used were as follows: BPA, bisphenol B (BBP), 4,4'-tetr-octylphenol, and 4-nonylphenol were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); 17β-estradiol (E2), tamoxifen, NADP, and glucose-6-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO); methoxychlor and chlorophenol red β-δ-galactopyranoside (CPRG) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and Boehringer Mannheim GmbH (Germany), respectively. SKF 525-A was a gift from Smith Kline & French Laboratories (Philadelphia, PA). BPA catechol and BPA α-quinone were synthesized according to the method reported by Atkinson and Roy (1995). Other chemicals used were of the highest quality commercially available.

*Recombinant yeast estrogenicity assay.* The recombinant yeast strain transfected with the human estrogen receptor (ERα) gene, together with expression plasmids carrying the reporter gene lac-Z preceded by an estrogen-responsive element (ERE) was provided with permission by Prof. Sumpter (Brunel University, U.K.). The yeast estrogenicity assay was conducted as described by Routledge and Sumpter (1996, 1997), with some minor modifications. In brief, to check an availability of the yeast cultured, 10-ml aliquots of ethanol solution of solid-phase extracts from incubation were transferred to 96-well plastic microtiter plates (Becton Dickinson, Falcon 3072 Microtest) to obtain dose-response curves. For an assay of estrogenic activity after incubation of EDCs with rat liver S9 fraction, 10-μl aliquots of ethanol solution of solid-phase extracts from incubation mixtures prepared as described below were transferred to wells. After evaporation of the solvent to dryness, 200-μl aliquots of the yeast assay medium containing recombinant yeast and CPRG, the chromogenic substrate of β-galactosidase reporter enzyme, were dispensed into each well. If needed, 1 μM tamoxifen was added to wells as an antiestrogen. The plates were sealed with 10-7 M as well as other EDCs tested, including BPA, ranging from 2.4 × 10−10 to 5 × 10−5 M as well as other EDCs tested, including BPA, ranging from 2.4 × 10−9 to 4.4 × 10−4 M were transferred to 96-well plastic microtiter plates (Becton Dickinson, Falcon 3072 Microtest) to obtain dose-response curves. For an assay of estrogenic activity after incubation of EDCs with rat liver S9 fraction, 10-μl aliquots of ethanol solution of solid-phase extracts from incubation mixtures prepared as described below were transferred to wells. After evaporation of the solvent to dryness, 200-μl aliquots of the yeast assay medium containing recombinant yeast and CPRG, the chromogenic substrate of β-galactosidase reporter enzyme, were dispensed into each well. If needed, 1 μM tamoxifen was added to wells as an antiestrogen. The plates were sealed with autoclave tape and incubated at 32°C in a dry incubator without shaking to avoid contamination between wells. After 24–48 h, the red color of the hydrolysis product of CPRG by β-galactosidase was read using a microplate reader (Bio-Rad, Model 550) in terms of the absorbance at 540 nm. All assays were carried out at least in duplicate using a blank well containing the yeast assay medium alone. The data were corrected for turbidity using the absorbance reading at 620 nm, and values were calculated as follows: Net OD540 = (OD540 for test − OD620 for test) − (OD540 for blank − OD620 for blank).

The concentration of EDC indicated, containing both the unchanged substrate and its metabolite(s), was expressed as a concentration of the substrate added in the respective incubation mixture.

**ERE-luciferase reporter assay in MCF-7 cells.** ERE-luciferase reporter assay using MCF-7 cells was carried out by the method described previously (Sugihara et al., 2000). In brief, MCF-7 cells in 12-well plates at 1 × 10⁴ cells/well were transiently transfected with 1.9 μg of pERE-luciferase reporter assay medium alone. The data were corrected for turbidity using the absorption reading at 620 nm, and values were calculated as follows: Net OD540 = (OD540 for test − OD620 for test) − (OD540 for blank − OD620 for blank).

The concentration of EDC indicated, containing both the unchanged substrate and its metabolite(s), was expressed as a concentration of the substrate added in the respective incubation mixture.

**HPLC analysis of BPA metabolites.** For an analytical HPLC, 40 μl of the sample solution prepared as above was applied to a Beckman Gold Nouveau HPLC system (Beckman Instruments, Inc., Fullerton, CA) equipped with a UV/VIS diode array detector and an analytical reversed-phase column (Supelcosil ABZ+ Plus; 4.6 × 150 mm, 5 μm; Supelco, Bellefonte, PA). For a preparative HPLC, 5 μl of the incubation mixture consisting of 250 mg liver equivalent of rat liver S9, the same final concentration of an NADPH-generating system as in the usual scale, and 0.5 mM BPA was incubated for 90 min at 37°C. The reaction was quenched by addition of 5 ml of 20% trichloroacetic acid (TCA), the quenched reaction mixture was allowed to stand for 15 min on ice, then was centrifuged at 2500 rpm for 10 min. The incubation system without an NADPH-generating system, or inactivated by addition of TCA prior to incubation, was used as control. A 1.8-ml aliquot of the resultant supernatant was passed through a Sep-Pak Plus C18 cartridge (Waters Associates Inc., Milford, MA) preconditioned with 10 ml methanol and 20 ml water for solid-phase extraction. The cartridge was washed with 10 ml water and the remaining water was purged by flushing with nitrogen gas. The adsorbed substances were eluted with 3 ml ethanol, and the eluate was evaporated to dryness in vacuo. The residue was dissolved in 200 μl ethanol, then 10 μl of the sample solution was transferred to a well of a microtiter plate for estrogenicity assay as described above. All the experiments were conducted in duplicate.

**Metabolic Activation of BPA by Rat Liver S9**

The estrogenic activity of BPA in the yeast assay was enhanced by incubation with rat liver S9 fraction more than three times compared with that of the control, in which the S9 was denatured with TCA prior to incubation (Fig. 1). This enhancement was partially inhibited by SKF 525-A, a well-
known inhibitor of cytochrome P450 (P450). The estrogenic activity generated with S9 was almost completely inhibited by inclusion of tamoxifen, an antagonist of estrogens, in the assay well. To test whether this enhancement of the estrogenicity is due to the increase of permeability of BPA into the yeast cell, which may be caused by the metabolite(s), we examined the effects of the extract containing metabolite(s) from the incubation mixture was assayed using a recombinant yeast screen. In the assay testing antagonist, 1 μM tamoxifen was added in the well of titer plate. Data represent the mean ± SE of four experiments.

FIG. 1. Metabolic activation of estrogenic activity of BPA by rat liver S9 fraction using yeast estrogenicity assay. In a complete system, BPA (0.1 mM) was incubated with native rat liver S9 fraction for 1 h at 37°C with or without 0.1 mM SKF 525-A, as described in the Materials and Methods section. In a denatured system, the incubation was carried out with the S9 fraction denatured by TCA prior to addition of BPA. The estrogenicity of solid-phase extracts from the incubation mixture was assayed using a recombinant yeast screen. In the assay testing antagonist, 1 μM tamoxifen was added in the well of titer plate. Data represent the mean ± SE of four experiments.

FIG. 2. Effects of combination of extracts from complete and denatured systems on estrogenicity by yeast estrogenicity assay. The indicated amounts of the solid-phase extracts in ethanol from the incubation system containing native (C) or denatured (D) S9 and 0.1 mM BPA were assayed by yeast screen as described in the Materials and Methods section. In some experiments, the extracts from complete and denatured systems were combined in the well and then assayed. Data represent the mean of two experiments.

increase the estrogenic activity (Fig. 4). The ability to activate the estrogenicity was restored by recombination of both microsomes and cytosolic fraction. It is noteworthy that although unchanged, BPA was decreased time-dependently by incubation with S9, but the estrogenicity of the extract was increased during incubation. Figure 6 shows the analytical HPLC profiles obtained by incubation of BPA with S9 or microsomes and cytosolic fraction alone. Peaks 1 and 2, eluted faster than unchanged BPA, were common metabolites by either S9 or microsomes, whereas no metabolite was formed with cytosol alone. The metabolite giving peak 2 showed a tendency to convert to that giving peak 1 during a storage of the extract at

FIG. 3. Effects of substrate concentration on metabolic activation of BPA by rat liver S9 fraction using yeast estrogenicity assay. Various concentrations of BPA were incubated with rat liver S9 fraction for 1 h at 37°C. The control incubation was carried out with the acid-denatured S9 fraction. The concentrations indicated are expressed as a substrate concentration in the incubation system. Other details are the same as in the legend to Figure 1. No error bar indicates the error was included within the symbol.
room temperature. On the contrary, peaks 3 and 4, eluted later than BPA, were detected only in the extract from S9 system, but from neither microsomes nor cytosolic fraction alone. Therefore, these results strongly suggest that the metabolite(s) giving either peak 3 or peak 4 must be a candidate as an active metabolite (see the following section).

Estrogenic Activity of BPA Metabolites

The preparative HPLC profiles of BPA metabolism catalyzed by rat liver S9 and the estrogenic activity of each fraction separated are shown in Figure 7. At least four metabolites, which were not found in the chromatogram of the acid-denatured control other than unchanged BPA eluted at 22.0 min, were detected in the extract from the native S9 system. Among them, peak 2, with a retention time of 20.7 min, was tentatively identified as authentic BPA catechol on the basis of its retention time on HPLC, UV spectrum ($
\lambda_{\text{max}}$ 280 nm), and $[\text{M-H}]^-$ = 242.98 by negative-mode LC/MS. Peak 1, eluted at a retention time of 18.7 min, was regarded as the secondary product of peak 2 because this peak increased with decreasing peak 2 during storage, as described above. Judging from its identity of the retention time and $[\text{M-H}]^-$ = 241.15 by negative-mode LC/MS with the compound found in the authentic
sample of BPA catechol, this metabolite might be BPA o-quinone. Peak 3 (retention time of 26.0 min) and peak 4 (retention time of 26.7 min), less-polar metabolites than BPA, seemed to be structurally isomeric compounds, showing almost the same mass numbers of [M-H]− 267.05 and [M-H]− 267.04, respectively, by negative-mode LC/MS. But the former metabolite gave two fragmented mass peaks of [M-H]− 251.85 and [M-H]− 132.85 by negative-mode LC/MS/MS, whereas the latter gave only [M-H]− 132.83. With respect to estrogenicity of these metabolites, only peak 4, not peak 3, exhibited a potent estrogenic activity comparable to that of BPA recovered (Fig. 7). Both BPA catechol and o-quinone showed no estrogenic activity at the concentrations tested. Thereby, the metabolite giving peak 4 should be an active metabolite as estrogen.

**Metabolic Modulation of Other EDCs by Rat Liver S9**

The effects of metabolism by rat liver S9 on the estrogenic activity of other EDCs, as well as E2, are shown in Figure 8. The metabolic activation of methoxychlor, which has no intrinsic estrogenic activity, by incubation with S9 was confirmed. BPB, 2,2-bis(4-hydroxyphenyl)butane, was also metabolically activated by S9. However, the estrogenic activities of 4-tert-octylphenol and 4-nonylphenol, as well as an endogenous estrogen, E2, were dramatically decreased by the S9-catalyzed metabolism.

**DISCUSSION**

The metabolic fate of BPA in adult rats is affected by many factors, such as the route of administration, the doses administered, the sex of rats (Pottenger *et al.*, 2000), and the strains used (Snyder *et al.*, 2000). In any case, the most predominant metabolite of BPA in rats in vivo has been attributed to the monoglucuronide of BPA (Knaak and Sullivan, 1966; Pottenger *et al.*, 2000; Snyder *et al.*, 2000). These observations were further supported by the evidence that BPA is rapidly converted to the glucuronide as a major metabolite in isolated rat hepatocytes (Nakagawa and Tayama, 2000). In contrast, however, Miyakoda *et al.* (1999; 2000) recently reported that about 50 ppb of free BPA was detected in fetuses 1 h after oral administration to the pregnant rats at a dose of 10 mg/kg. In this case, no glucuronide was detected in the fetuses, whereas the glucuronide was again a major metabolite in the dam. These results suggest that BPA easily passes through the placental barrier and the fetus might lack the ability of glucuronide formation. Subsequently, in fact, Yokota *et al.* (2000) have demonstrated that the activity of UGT2B1, a predominant isoenzyme to catalyze the glucuronidation of BPA (Yokota *et al.*, 1999), was not detected in the fetus of rat. It is true that the rodent fetal liver has a limited ability to metabolize xenobiotics compared to the adult liver. Currently available data, however, revealed that several forms of P450 are expressed in fetal rat liver, especially in late gestation (Cresteil *et al.*, 1986; Neubert and Tapken, 1988; Raucy and Carpenter, 1993). In addition, unlike rodent species, the human fetal liver has a significant capacity for xenobiotic metabolism (Hakkola *et al.*, 1998; King...
et al., 1999). With regard to the glucuronosyltransferase activity in human fetal liver, there is an interesting report that the glucuronidation of ritodrine, a β2-adrenoceptor having a bisphenol structure similar to BPA, is little developed compared to the adult liver (Pacifci et al., 1993). Therefore, to understand the effects of BPA on the fetus, a target of EDCs, it is indispensable to investigate the metabolism of BPA other than glucuronidation.

Rat liver S9 fraction is often used as the source of the microsomal P450-dependent monoxygenase system for metabolic activation of promutagens in the Ames assay (Ames et al., 1975). Therefore, the rat liver S9 fraction was also adopted in this experiment to examine the effects of metabolic conversion of EDCs on their estrogenic activity. When BPA was incubated with rat liver S9, its estrogenic activity assessed by the yeast reporter assay increased with both a time- and substrate concentration-dependent manner (Figs. 3 and 4). Based on the result showing no synergistic increase of estrogenicity from the combination of the extracts, compared to the complete and denatured systems in the yeast assay, it seems very likely that the enhanced activity is due to the formation of true estrogen metabolite(s), but not to the metabolite(s) causing an increase of permeability of BPA which remained across the yeast cell wall (Fig. 2). Furthermore, this metabolic activation was also confirmed by the results obtained with breast cancer cell line MCF-7 cells (Fig. 5). Similar metabolic activation of BPA was also observed by using human liver S9 (data not shown). The active metabolite contributing to the enhancement of estrogenicity was eluted at a retention time later than unchanged BPA on a reversed-phase HPLC (Figs. 6 and 7). Judging from the peak sizes detected, if the extinction coefficient of the unknown metabolite is similar to that of BPA, the estrogenicity of this minor metabolite must be much more intense than that of a parent BPA (Fig. 7). Unfortunately, with respect to the structure of this active metabolite, at present little is known except a) its molecular weight might be 40 mass greater than that of BPA; b) it gives a mass peak of [M-H] = 132.83, suggesting a dimer of propenylphenol structure on negative-mode LC/MS/MS; and c) it possesses two hydroxyl groups to be trimethylsilylated (data not shown). Much more interesting evidence about this active metabolite is that the formation of this metabolite required both microsomes and cytosol (Figs. 4 and 6). In this metabolic conversion, because the reaction required NADPH and was inhibited with SKF 525-A, microsomal P450 should be involved at least as a primary enzyme. The function of cytosol was lost by boiling but still retained after dialysis (data not shown), suggesting an involvement of certain enzyme.

It is not clear, however, whether such a factor in cytosol acts as a secondary enzyme, converting the primary metabolite by microsomal P450 to the active one. Atkinson and Roy (1995) have demonstrated that DNA adduct formation in vitro with BPA is catalyzed by rat liver microsomes only in the presence of NADPH. Based on these results, they speculated that BPA might be activated by P450 to form a reactive BPA o-quinone via 5-hydroxy BPA (BPA catechol). We first demonstrated directly the formation of these monooxygenated metabolites, but they were almost inactive as an estrogen (Fig. 7). We could not identify a peak corresponding to 4,4′-dihydroxy-α-methylstilbene, an intermediary metabolite in bacteria (Sivivack et al., 1994) that exhibits estrogenicity about 100 times more potent than that of BPA (our unpublished data).

BPB, a weak estrogen having a quite similar structure with BPA, was also activated by rat liver S9 (Fig. 8). The HPLC profile of BPB metabolites was similar to that of BPA metabolites (data not shown), indicating the same type of metabolic activation might occur. We also reconfirmed the metabolic activation of methoxychlor, which is known to be activated by O-demethylation to 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane by P450 (Bulger et al., 1978). In contrast, 4-tert-octylphenol and 4-nonylphenol, alkylphenolic EDCs widely distributed in the environment, as well as an endogenous estrogen, E2, were metabolically inactivated in terms of estrogenicity by S9 (Fig. 8).

In conclusion, both BPA and BPB are metabolically activated in terms of estrogenicity by rat liver S9 fraction. This might account, at least in part, for the discrepancy between the estrogenic activities of BPA observed in vitro and in vivo (Steinmetz et al., 1997). Even though the major metabolic route of BPA in vivo could be glucuronidation rather than P450-dependent metabolism (Pottenger et al., 2000; Snyder et al., 2000), the formation of small amounts of unconjugated metabolites have been demonstrated in vivo (Knaak and Sullivan, 1966; Pottenger et al., 2000) and in vitro (Nakagawa and Tayama, 2000). The most intensive enhancement, 22.8 ppm, was observed at a substrate concentration of 0.1 mM BPA. At the concentrations lower than 0.01 mM BPA (2.28 ppm), which are comparable to the values reported in vivo Cmax levels in the blood of female rats following administration of 100 mg/kg (Pottenger et al., 2000), there was no enhancement of estrogenic activity. However, the metabolic activation by S9 must be taken into account in order to assess BPA as an in vivo estrogen in the fetus, one of the most important targets of EDCs, because the fetus has a poor ability to conjugate BPA with glucuronic acid (Miyakoda et al., 1999; 2000; Yokota et al., 2000) as a detoxification reaction. Further studies on the mechanism of this unique metabolic activation of BPA by S9, as well as the structural elucidation of the active metabolite, are in progress.

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REFERENCES


