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Elevated Serum Bisphenol A Levels under Hyperandrogenic Conditions may be Caused by Decreased UDP-glucuronosyltransferase Activity

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Abstract. This study was performed to investigate the effect of androgen on the metabolism of bisphenol A (BPA), an endocrine disruptor, in order to clarify the mechanism of the higher levels of serum BPA in men and hyperandrogenemic women compared with normal women. Castrated female rats (OVX) were subcutaneously injected with testosterone propionate (TP) (0.01, 0.1, and 1 mg) every day for 2 weeks. Serum BPA concentrations in OVX rats showed a TP dose-dependent increase and were significantly higher at 0.1 and 1.0 mg of TP. The enzyme reaction of BPA glucuronidation in the rat liver microsomes showed that the ratio of glucuronide in the OVX rats was significantly reduced in a TP dose-dependent manner. Analysis of the mRNA expression of UDP-glucuronosyltransferase 2B1 (UGT2B1) by real-time quantitative RT-PCR revealed that the relative expression level of UGT2B1 mRNA showed a TP dose-dependent decrease. The results of enzyme analyses demonstrated that the ratio of BPA glucuronidation and the expression level of UGT2B1 mRNA were significantly lower under the hyperandrogenemic conditions. The clearance of BPA may be slowed in a TP dose-dependent manner, resulting in an increase of serum BPA concentration under hyperandrogenemic conditions.

Key words: Bisphenol A, UDP-glucuronosyltransferase, UGT2B1, Endocrine disruptor, Androgen

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THERE is substantial evidence that endocrine-disrupting chemicals have the potential to alter the normal function of the endocrine system in wildlife and humans [1, 2]. Various environmental chemicals are known to act as endocrine disruptors, which have been reported to exhibit estrogenic, anti-estrogenic and/or anti-androgenic actions in wildlife and human beings. There is increasing scientific concern and public debate about the involvement of endocrine-disrupting chemicals in a number of human health disorders.

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Bisphenol A (BPA), an estrogenic endocrine-disrupting chemical with two unsaturated phenol rings, is widely used in the production of polycarbonate plastics and epoxy resins, which are used in dentistry, food packaging, and as lacquers for coating food cans, bottle tops and water pipes [3, 4]. A significant amount of BPA has been detected in liquid from canned vegetables that are exposed to high temperature during autoclaving [5], and in the saliva of dental patients fitted with restorative materials [6]. BPA has been reported to bind to estrogen receptors (ERα and ERβ) and to play either estrogenic or anti-estrogenic roles *in vitro* [7, 8]. BPA has been shown to exhibit several actions, such as uterotrophic effects [9], decreasing sperm production [10], stimulation of prolactin release [11], promotion of cell proliferation in a breast cancer cell

line [5], and influencing preimplantation development [12], in animal experiments. Promotion of growth and puberty by fetal or preimplantation exposure to BPA in mice has been reported [13, 14]. We reported that BPA was detectable not only in the serum but also in several biological fluids, including amniotic fluid, demonstrating human fetal exposure to BPA through the placenta [15]. It is interesting to note that there was a gender difference in serum BPA concentrations in humans [16] and, more recently, determination of serum BPA concentrations in women with or without ovarian dysfunction revealed that BPA levels may vary according to the endocrinological status of subjects [17]. BPA levels in patients with endometrial hyperplasia and endometrial cancer, both of which are estrogen-related uterine disorders, were lower compared to those in normal controls [18]. These findings suggest an association between BPA exposure and endocrine disorders. The mode of action of BPA may be even more complex than expected, but analysis of BPA metabolism in the body may provide clues about the mechanisms of linkage between the occurrence of estrogen-related diseases and endocrine disruption.

The glucuronidation reaction is catalyzed by members of the UGT enzyme superfamily [19]. The UGT enzymes are classified into two families, UGT1 and UGT2, on the basis of amino acid sequence homology; the latter is further subdivided into the subfamilies UGT2A and UGT2B. Moreover, UGT2B is subdivided into many isoenzymes. Each isoenzyme has specific substrate(s) for glucuronidation. BPA is known to be glucuronidated by liver microsomes in a reaction catalyzed by UGT2B1, and then to be rapidly excreted in the feces and urine [20–22]. We reported that the ratio of BPA glucuronidation and the expression level of UGT2B1 mRNA were significantly higher in female than in male rat livers [23].

In this study, we investigated the effect of androgen on the metabolism of BPA in rats, in order to clarify the mechanism of the sex difference in serum BPA levels and of the higher levels of serum BPA in patients with polycystic ovary syndrome compared with normal women.

Materials and Methods

Reagents

Bisphenol A (>95% pure) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Testosterone propionate, uridine 5'-diphosphoglucuronic acid, Tris-HCl buffer, bovine serum albumin (BSA), EDTA, sodium carbonate, sodium citrate, and Folin-phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sucrose was obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). Magnesium chloride, potassium chloride, methanol, ethanol, copper sulfate pentahydrate, β-glucuronidase, and dimethylbutylidene-bisphenol (internal standard for HPLC) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Acetonitrile and acetic acid were purchased from Kanto Kagaku Co. (Tokyo, Japan).

Animals and their experimental preparation

Eight-week-old female Wistar-Imamichi rats, which were obtained from Saitama Experimental Animals Supply Co., Ltd. (Saitama, Japan), were castrated. Castrated female rats (OVX) were subcutaneously injected with testosterone propionate (TP) (0.01 mg, 0.1 mg, 1 mg; n = 5, respectively) in 0.5 ml of corn oil every day for 2 weeks starting on the fourth day after the castration. The experimental groups consisted of intact rats, ovariectomized rats without TP (OVXTP-0), ovariectomized with TP 0.01 mg (OVXTP-0.01), ovariectomized with TP 0.1 mg (OVXTP-0.1) and ovariectomized with TP 1 mg (OVXTP-1). The rats in intact control and OVXTP-0 groups were subcutaneously injected with 0.5 ml of corn oil. The rats were housed in a controlled-climate room at 22 ± 2°C and 62.5 ± 2.5% relative humidity, with lights on from 08.00 to 20.00 h. They were allowed free access to a standard rodent diet and tap water in glass bottle. Wood chips were used as bedding. Blood samples were obtained by decapitation, and all sera were stored at –30°C until assayed. Livers were removed promptly and stored at –80°C until used.

Serum BPA concentrations in each group of animals (n = 5, respectively) were determined before and 120 min after subcutaneous injection with BPA in corn oil (50 mg/kg). In some experiments, serum BPA concentrations were measured before and at 60, 120, 180 min after the treatment with BPA.

Measurement of rat serum BPA, testosterone and estradiol concentrations

Rat serum BPA concentrations were assayed with a

competitive ELISA [24]. This assay method measures total (conjugated and unconjugated) BPA. Serum testosterone and estradiol concentrations were assayed with ^{125}I-RIA kits (Diagnostic Products Corporation, Los Angeles, CA, USA). All assays were performed in duplicate, and all of the samples were assayed simultaneously. The intra-assay coefficient of variation was less than 10%.

Preparation of liver microsomes and enzyme activity analysis

Livers were perfused with 5 ml of ice-cold physiological saline, and homogenized with five volumes of 50 mM Tris/HCL buffer (pH 7.5) containing 0.25 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$, using a glass-teflon homogenizer. The homogenates were centrifuged at 1000 g for 10 min, and the supernatants were centrifuged at 12000 g for 20 min, and the resultant supernatants were centrifuged at 105000 g for 60 min. The cytosolic supernatants were removed, and the remaining microsomal pellets were suspended in 50 mM Tris/HCl buffer containing 0.25 M sucrose, and stored at –80°C until used. The protein content was determined by the method of Lowry *et al.* [25] using BSA as a standard.

One milligram of microsomal protein was incubated in a total volume of 2.0 ml of 0.1 M Tris/HCl buffer (pH 7.2) containing 1 mM BPA, 0.1 mM UDP-glucuronic acid, 10 mM $MgCl_2$, and 20 μM EDTA. Reactions were performed at 37°C with shaking (frequency: 100 cycles/min; amplitude: 3.5 cm) for 30 min. The reaction was terminated by the addition of 100 μl of methanol, and proteins were removed by centrifugation at 1000 g for 15 min. The supernatants were stored as the reaction products at –80°C until assayed.

Measurement of glucuronide fraction of BPA in the microsomal reaction products

The microsomal reaction supernatant sample was divided in two aliquots for measuring total and unconjugated BPA. After 100 μl of the supernatant sample was washed with 1 ml of a mixture of n-hexane and ethylether (3 : 2), the unconjugated fraction was eluted in the solvent. An aliquot (20 μl) of the solvent fraction was evaporated under a stream of nitrogen gas. An aliquot (1.25 ml) of 0.1 M Tris/HCl buffer containing 1 M $MgCl_2$ and 0.5 mM EDTA was added to the dry residue in the test tube. This sample was used for assaying unconjugated BPA. The procedure for the measurement of total BPA was done as follows. The above-mentioned Tris buffer (1 ml) was added to an aliquot (100 μl) of the supernatant sample, and then 1.23 ml of the Tris buffer was added to 20 μl of the mixture. This sample was used for assaying total BPA by ELISA, which measured both conjugated and unconjugated BPA. This method was selected after comparison with an HPLC technique [23]. There was a significant correlation ($r = 0.877$, $p<0.001$) in the results between this extraction method and the HPLC assay.

RT-PCR analysis of UGT2B1 mRNA

Real-time quantitative RT-PCR was used for the quantification of UGT2B1 mRNA. In brief, 1 μg of total RNA was prepared from rat liver using a modified acid guanidium thiocyanate-phenol-chloroform method. The total RNA treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) was subjected to reverse transcription using oligo dT 20 (Takara, Tokyo, Japan) and superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) at 42°C for 30 min, followed by RNase H treatment. Aliquots of the cDNA (1/20) were used as templates for real-time PCR analysis using a LightCycler system (Roche, Mannheim, Germany). The PCR program consisted of 40 cycles of 8 sec at 94°C, 5 sec at 60°C, and 10 sec at 72°C. Oligonucleotide primers for UGT2B1 were as follows: forward, 5'-TGTTGGTAT TCCCTTGTTTGC-3'; reverse, 5'-GTGCTTGGCTC CTTTGTGACG-3'. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), housekeeping gene, were as follows: forward, 5'-ACCACAGTC CATGCCATCAC-3'; reverse, 5'-TCCACCACCCT GTTGCTGTA-3'. The RNA preparation and RT-PCR in the present study were performed in triplicate.

Statistical analyses

All results are expressed as means ± standard errors of the mean (SEM). Statistical comparisons among the groups were performed by analysis of variance (ANOVA) and the least significant difference test (LSD). Correlation coefficients were calculated by linear regression analysis. Significance was taken at $p<0.05$.

Results

Table 1 shows serum BPA, testosterone and estradiol concentrations in the intact and OVX rats. The serum BPA concentrations were significantly higher in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group. The serum testosterone concentrations were decreased by half in the OVXTP (0) control rats, and were significantly higher in a dose-dependent manner in OVX rats treated with TP. The serum estradiol concentrations in the OVX rats were decreased to less than half the control levels. There was a significant positive correlation between the serum BPA and testosterone concentrations ($r = 0.983$, $p<0.001$), but not between the serum BPA and estradiol concentrations ($r = –0.032$, NS) in all rats.

Subcutaneous injection of BPA (50 mg/kg) into intact female rats caused an abrupt increase of serum BPA concentrations, which reached a peak (749.6 ± 113.8 ng/ml) at 120 min after the treatment, as shown in Fig. 1. Castrated female rats were also subcutaneously injected with BPA (50 mg/kg), and blood samples were obtained at 120 min after the treatment with BPA. The serum BPA concentrations increased in a TP dose-dependent manner, and were significantly higher in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group (Fig. 2).

The levels of total, unconjugated, and conjugated BPA, and the ratios of conjugated to total BPA in the supernatant of the rat liver microsome reaction are listed in Table 2. There were no differences in the total levels of BPA in the reaction products among the groups. The ratio of conjugated to total BPA was significantly lower ($P<0.05$) in the OVXTP (0.1) and OVXTP (1) groups than in the OVXTP (0) or intact control group.

The relative expression level of GAPDH mRNA was not significantly different among the groups, but that of UGT2B1 mRNA was significantly lower ($P<0.05$) in

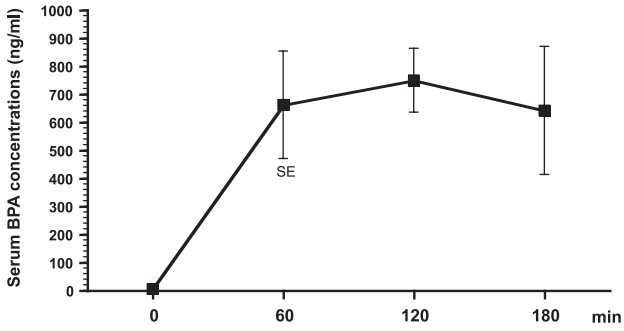

Fig. 1. Serum BPA concentrations in intact female rats after subcutaneous injection of BPA (50 mg/kg).
Subcutaneous injection of BPA into intact female rats caused an abrupt increase of serum BPA concentrations, which reached a peak at 120 min after the treatment (n = 5, respectively).

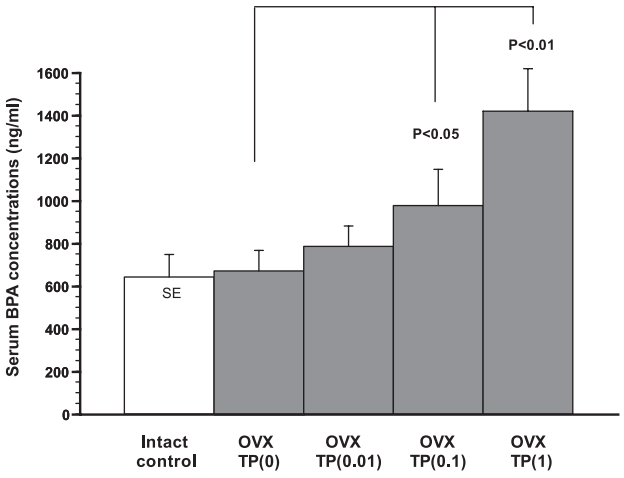

Fig. 2. Serum BPA concentrations in female intact control, ovariectomized (OVX) control, and OVX rats with testosterone propionate (TP) at 120 min after subcutaneous injection of BPA (50 mg/kg).
The serum BPA concentrations increased in a TP dose-dependent manner, and were significantly higher in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group. Values are mean ± SEM (n = 5 in each group).

Table 1. Basal serum BPA, testosterone and estradiol concentrations in intact and ovariectomized (OVX) rats

	Intact control	OVXTP (0)	OVXTP (0.01)	OVXTP (0.1)	OVXTP (1)
BPA (ng/ml)	4.38 ± 0.34	4.10 ± 0.42	4.51 ± 0.58	5.34 ± 0.44[a,c]	5.76 ± 0.50[a,c]
Testosterone (ng/ml)	0.65 ± 0.15	0.31 ± 0.05[a]	1.27 ± 0.66	28.11 ± 4.06[b,d]	46.90 ± 17.9[b,d]
Estradiol (pg/ml)	40.0 ± 11.7	15.0 ± 2.0[a]	11.3 ± 1.3[a]	14.0 ± 4.0[a]	18.3 ± 4.1[a]

Data are mean ± SEM (n = 5 in each group). TP (0.01, 0.1, 1): Testosterone propionate (0.01, 0.1, 1 mg)
a; $P<0.05$, compared with intact control b; $P<0.01$, compared with intact control
c; $P<0.05$, compared with OVXTP (0) d; $P<0.01$, compared with OVXTP (0)

Table 2. The levels of total, unconjugated and conjugated BPA and the ratios of conjugated to total BPA in the supernatant after microsomal enzyme reaction

BPA	Intact control	OVXTP (0)	OVXTP (0.01)	OVXTP (0.1)	OVXTP (1)
Total (ng/ml)	10.53 ± 0.22	10.11 ± 0.20	10.29 ± 0.20	10.31 ± 0.05	10.60 ± 0.50
Unconjugated (ng/ml)	5.80 ± 0.61	5.85 ± 0.20	6.68 ± 0.82	7.02 ± 0.40	7.23 ± 0.57
Conjugated (ng/ml)	4.72 ± 0.57	4.26 ± 0.55	3.60 ± 0.95	3.30 ± 0.44	3.26 ± 0.26
Ratio of conjugated to total	0.448 ± 0.049	0.421 ± 0.045	0.350 ± 0.087	0.320 ± 0.041[a,b]	0.306 ± 0.031 [a,b]

Data are mean ± SEM (n = 5 in each group).
a; P<0.05, compared with intact control b; P<0.05, compared with OVX control

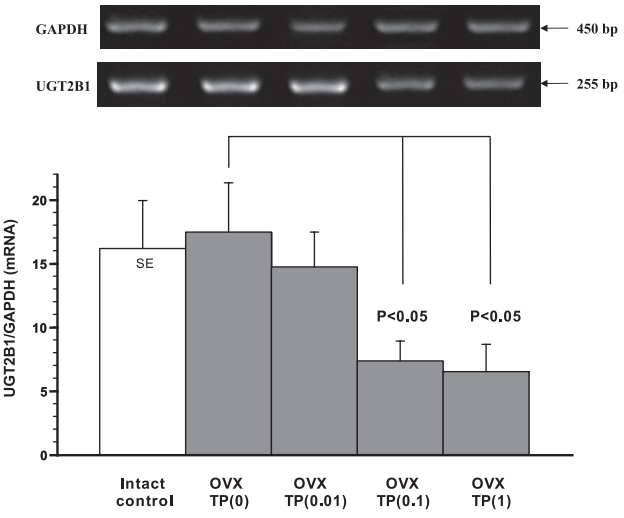

Fig. 3. Expression of UGT2B1 mRNA in the livers of female intact control, ovariectomized (OVX) control, and OVX rats treated with testosterone propionate (TP).
The relative expression level of UGT2B1 mRNA was significantly lower in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group. Values are mean ± SEM (n = 5 in each group).

the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group (Fig. 3). The bands of GAPDH and UGT2B1 were detected as expected by electrophoretic analysis of the RT-PCR products, which were collected before the reaction plateau levels.

Discussion

BPA was detectable in all serum samples from rats. It was reported that BPA was found at a concentration of 0.6 ± 0.1 ng/ml in tap water in Korea [26]. We also detected 0.11–0.21 ng/ml of BPA in the tap water in our animal-breeding room (unpublished data). The main source of BPA consumed by the animals may be tap water, and the amount of BPA taken may be approximately 2–8 ng/day assuming that each rat drinks 20–40 ml of water. There was no difference in the volume of tap water drunk by the various groups of rats. Serum BPA concentrations in OVX rats with TP treatment showed a dose-dependent increase and were significantly higher at 0.1 and 1.0 mg of TP. The ratio of BPA glucuronidation in the OVX-rat liver microsome reaction was significantly lower in a TP dose-dependent manner. The clearance of BPA may be slowed in a TP dose-dependent manner, resulting in an increase of serum BPA concentrations with testosterone dose. Moreover, the relative expression level of UGT2B1 mRNA showed a TP dose-dependent decrease. These findings suggest that the amount of UGT2B1 enzyme catalyzing BPA glucuronidation is decreased by treatment with TP. To our knowledge, this is the first report of an effect of androgen on UGT2B1 enzyme.

In human beings, serum BPA concentrations are significantly higher in men than in women [16], and also in hyperandrogenemic women compared with normal women [17]. Moreover, we reported that there were significant positive correlations between serum BPA and total testosterone, free testosterone, androstenedione, and DHEAS concentrations in all female subjects [17]. Rat serum BPA concentrations are also significantly higher in males than in females, as in humans [23]. These *in vivo* data may implicate the relation of serum BPA concentrations with the androgen levels and are in accord with the present results.

Glucuronidation represents one of the conjugation reactions for the inactivation and excretion of both endobiotic and xenobiotic compounds. It is known that the metabolites of BPA include a monoglucuronide, a sulfate conjugate, and a glucuronide/sulfate diconjugate. Pritchett *et al.* [27] demonstrated that BPA-glucuronide was the major metabolite formed by hepatocytes of humans, rats and mice. The amount of BPA-sulfate was

much smaller (0–2%) than that of BPA-glucuronide, and especially, the former was not detected in female rats. The liver is a major site of glucuronidation as evidenced by the quantitative analysis. However, extrahepatic glucuronidation is also known to occur in organs such as the lung, kidney, gastrointestinal mucosa, prostate and olfactory epithelium [28–33]. The overall quantitative contribution of extrahepatic glucuronidation is much smaller compared with hepatic glucuronidation. Accordingly, glucuronidation in the liver influences the serum levels of both endobiotic and xenobiotic compounds. Extrahepatic glucuronidation might play an important role in the local regulation of the effects of compounds on various organs or tissues. In the present study, we investigated the conjugation of BPA in rat liver microsomes, and found that the conjugation rate of BPA was significantly lower under hyperandrogenemic conditions.

In conclusion, the results of enzyme analyses demonstrated that the ratio of BPA glucuronidation and the expression level of UGT2B1 mRNA were significantly lower under hyperandrogenemic conditions. The clearance of BPA is slowed concomitantly with the elevation of serum testosterone levels, resulting in an increase of serum BPA concentrations. The gender difference of serum BPA concentrations and the higher concentrations of BPA in hyperandrogenemic women may be due to the difference of androgenecity. The findings in this study stress the importance of the metabolism of endocrine disruptors.

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