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Bisphenol A Binds to Protein Disulfide Isomerase and Inhibits Its Enzymatic and Hormone-Binding Activities

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Bisphenol A [2,2-bis-(4-hydroxyphenyl) propane; BPA] is a versatile industrial material for plastic products, but is increasingly being recognized as a pervasive industrial pollutant as well. Accumulating evidence indicates that the environmental contaminant BPA is one of the endocrine-disrupting chemicals that potentially can adversely affect humans as well as wildlife. To define the molecular aspects of BPA action, we first investigated the molecules with which it physically interacts. High BPA-binding activity was detected in the P2 membrane fraction prepared from rat brains. As determined by SDS-PAGE analysis, the molecular mass of a BPA-binding protein purified from the rat brain P2 fraction was 53 kDa. The N-terminal amino acid sequence of the purified BPA-binding protein was identical with that of the rat protein disulfide isomerase (PDI), which is a multifunctional protein that is critically involved in the folding, assembly, and shedding of many cellular proteins via its isomerase activity in addition to being considered to function as an intracellular hormone reservoir. The K_d value of BPA binding to recombinant rat PDI was 22.6 ± 6.6 μM. Importantly, the binding activity of L-T_3 and 17β-estradiol hormones to PDI was competitively inhibited by BPA in addition to abolishing its isomerase activities. In this paper we report that the ubiquitous and multifunctional protein PDI is a target of BPA and propose that binding to PDI and subsequent inhibition of PDI activity might be mechanistically responsible for various actions of BPA. (*Endocrinology* 147: 2773–2780, 2006)

BISPHENOL A [2,2-bis-(4-hydroxyphenyl) propane; BPA] is widely used as a monomer for plastic products, including polycarbonate and other epoxy resins, which are used in the coating of food cans, dental sealants, *etc.* (1–3). BPA and several chlorinated derivatives of it are commonly found in industrial waste effluents, including those from paper-manufacturing plants (4), often leading to the contamination of ground water. As such, BPA and its derivatives are common pollutants of rivers, lakes, and seawater, resulting in chronic exposure of humans and wildlife to BPA. In fact, BPA has been detected in the sera and placentas of pregnant women as well as in amniotic fluid (5, 6). High concentrations of BPA (30 μg/ml; 131 μM) also have been detected in saliva after dental treatment (7). BPA is considered to be one of the most widespread endocrine-disrupting chemicals (EDCs), and recently, its adverse effects on human health and wildlife are being increasingly recognized.

Mounting evidence from numerous studies of BPA reveals that BPA has diverse influences on various physiological functions related to steroid hormones (8–11), thyroid hormones (12, 13), the nervous system (14–18), the immune system (19, 20), and other cell signaling pathways (21, 22). For example, BPA possesses estrogenic and antiandrogenic activities *in vitro* (1–3, 23, 24) and influences reproductive functions, sexual differentiation, and behavioral patterns *in vivo* (8–11, 17). BPA is also demonstrated to antagonize L-T_3 (T_3) action *in vitro* (12, 25). In Sprague Dawley rats, dietary exposure to BPA during pregnancy and lactation causes an increase in the serum total T_4 in pups postnatally (12). Prenatal and neonatal exposures of mice to BPA activate aggressive behavior (18), enhance dopamine D1 receptor-dependent rewarding effects induced by psychostimulant methamphetamines (14), and cause up-regulation of immune responses, especially T helper 1 responses in adulthood (19). Furthermore, BPA induces dopamine release in a nongenomic manner through guanine nucleotide-binding proteins and N-type calcium channels in cultured cells (15).

The actions of BPA *in vivo* and *in vitro* are very diverse and multiple, as indicated above. However, very little is known about the target molecules of BPA and the molecular mechanisms responsible for mediating its effects. As for the influence of BPA on steroid sex hormones, it has been demonstrated that BPA binds to nuclear estrogen receptor (ER) and acts as an agonist and also binds to human SHBG (hSHBG) and thereby disturbs the binding of endogenous sex hormones to hSHBG (23, 24, 26, 27). In addition, Niwa *et al.* (28) have shown that BPA competitively inhibits the activity of steroidogenic cytochrome P45017 (CYP17), suggesting that BPA also interferes with the biosynthesis of steroid hormones. These findings collectively support the idea that BPA shows estrogenic activity and interferes with the homeostasis of sex hormones such as 17β-estradiol (E_2) and testosterone. As for the influence of BPA on thyroid hormone, it has been shown that BPA binds to nuclear thyroid hormone receptor (TR) and acts as an antagonist in addition to binding to transthyretin (TTR) (25, 29). However, the affinity of BPA to TR seems to be relatively low at 200 μM, and the major T_4 carrier protein in humans is T_4-binding globulin, which ac-

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Abbreviations: BPA, Bisphenol A; B_{max}, maximal binding capacity; CYP17, cytochrome P45017; E_2, 17β-estradiol; EDC, endocrine-disrupting chemical; ER, estrogen receptor; h, human; InB, oxidized insulin chain B; P, pellet; PDI, protein disulfide isomerase; RNase, ribonuclease; rr, recombinant rat; S, supernatant; TR, thyroid hormone receptor; TTR, transthyretin.

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counts for 70–80% of thyroid hormone-binding activity, but not TTR, implying that the influence of BPA binding to TTR is of limited importance in humans. Thus, at present, despite BPA's various *in vitro* and *in vivo* activities, its target proteins remain obscure and ill defined.

In this study we focused our efforts on exploring and defining target molecules of BPA. We demonstrate by direct binding studies that protein disulfide isomerase (PDI; EC 5.3.4.1) is a novel target of BPA and also provide evidence indicating that the binding of BPA to PDI results in the disruption of PDI actions, which could, in turn, adversely affect many cellular processes.

Materials and Methods

Materials

BPA was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). [G-^{3}H]BPA ([^{3}H]BPA; specific activity, 5 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [^{125}I]T_3 (specific activity, 97.3 Ci/mmol) and [2,4,6,7-N-^{3}H]E_2 ([^{3}H]E_2; specific activity, 95 Ci/mmol) were products of PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA). Sucrose monolaurate was purchased from DOJINDO Laboratories (Kumamoto, Japan). T_3, E_2, protease inhibitor cocktails, bacitracin, oxidized insulin chain B (InB), and ribonuclease A (RNase) type III from bovine pancreas were purchased from Sigma-Aldrich Corp. (St. Louis, MO). All other chemicals were obtained from Wako Pure Chemical Industries Ltd. BPA and E_2 were dissolved in ethanol at 100 mM to make stock solutions and were stored at −20 C. T_3 was dissolved in 0.1 M NaOH at 10 mM to make stock solutions and was stored at −20 C. Appropriate vehicle controls were performed in all experiments.

Preparation of subcellular fractions from rat brains

All steps in this procedure were carried out at 4 C. Adult male rats (Sprague Dawley; 6 wk old; CLEA Japan, Inc., Tokyo, Japan) were killed by decapitation. Whole brains were harvested and rinsed in ice-cold homogenate buffer [10 mM Tris-HCl buffer (pH 7.5) including 0.32 M sucrose and 0.05% protease inhibitor cocktail] to eliminate blood and other debris. The brains were homogenized in 9 vol (wt/vol) of the homogenate buffer by 10 strokes at 900–1000 rpm using a Teflon-glass homogenizer, then the brain homogenate was centrifuged at 1500 × *g* for 10 min. The supernatant (S1) was subjected to an additional centrifugation step at 17,500 × *g* for 15 min. The pellet obtained (P2) was suspended in the homogenate buffer and washed by centrifugation at 17,500 × *g* for 15 min. The supernatant (S2) was centrifuged at 100,000 × *g* for 60 min and separated into supernatant (S3) and pellet fractions (P3). The pellet (P3) was then suspended in homogenate buffer and washed by centrifugation at 100,000 × *g* for 60 min.

Radioligand binding assay

The P2 membrane fraction (50 μg protein) was incubated with radioligands ([^{3}H]BPA, [^{125}I]T_3, or [^{3}H]E_2) in 0.5 ml binding buffer [50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl] for 2 h at 4 C. The reaction mixture was centrifuged at 14,500 rpm for 5 min at 4 C. Pellets were washed twice in ice-cold binding buffer and dissolved in 0.2 ml 0.1 M NaOH. In the experiments using subcellular fractions prepared from rat brains, the reaction mixture was aspirated rapidly through a GF/B filter (Whatman, Middlesex, UK), and the filter was washed three times with 3 ml ice-cold binding buffer. In the case of purified recombinant PDI, proteins in the reaction mixture were precipitated by adding 0.5 ml binding buffer including 12% polyethylene glycol 6000 and 0.2 M $ZnCl_2$. After centrifugation at 14,500 rpm for 5 min at 4 C, the pellets were washed twice in ice-cold binding buffer including 6% polyethylene glycol 6000 and 0.1 M $ZnCl_2$ and finally dissolved in 0.2 ml 0.1 M NaOH. The radioactivity in each sample was countered in Pico-Fluor 40 scintillation cocktail (PerkinElmer, Norwalk, CT) using a β-counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA) or a γ-counter (auto well γ-counter ARC-2000, Aloka Co. Ltd., Tokyo, Japan). Nonspecific binding was defined in the presence of 1 mM unlabeled BPA, 30 μM unlabeled T_3, or 100 μM unlabeled E_2. Specific binding was defined as bound radioactivity, calculated by subtracting nonspecific from total binding. In the case of competitive binding assay, competing ligands were added to the reaction mixture. Saturation studies were performed with nine concentrations of [^{3}H]BPA.

Preparation of BPA affinity resin

BPA amine derivative (Fig. 1) was a gift from Kobe Natural Products and Chemicals Co. Ltd. (Hyogo, Japan). BPA-Sepharose resin was prepared by coupling BPA amine derivative to cyanogen bromide-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Purification of BPA-binding proteins from rat brains

All steps were carried out at 4 C. Rat brain P2 fraction (250 mg proteins) was solubilized in homogenate buffer containing 0.5% sucrose monolaurate with gentle stirring for 60 min and then centrifuged at 100,000 × *g* for 60 min. The supernatant was loaded onto an anion ion exchange Whatman DE52 column (2.5 × 5.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0; buffer A). The proteins were eluted stepwise with 12.5 ml buffer A containing 0.1, 0.2, or 0.5 M NaCl. The BPA-binding activity and protein concentration in each fraction were measured. The fraction, which had high [^{3}H]BPA binding activity, was concentrated by ultrafiltration using Amicon (Millipore Corp., Billerica, MA). The concentrated fraction was incubated for 2 h with gentle rotation in 2 ml BPA-Sepharose affinity resin equilibrated with buffer A. BPA-Sepharose affinity resin was washed in 50 mM Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl (buffer B), and bound proteins were eluted with gentle mixing for 1 h in buffer B containing 2 mM BPA.

Cloning and expression of rat PDI

Total RNA was extracted from a rat brain (Sprague Dawley; male; 6 wk old; CLEA Japan, Inc.) using total RNA isolation reagent (Isogen, Nippon Gene, Toyama, Japan). After deoxyribonuclease treatment (Nippon Gene) to remove contaminating genomic DNA, total RNA was reverse transcribed using an RNA PCR kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The reaction mixture, containing 5 U reverse transcriptase avian myeloblastosis virus, 1× RT-PCR buffer, 1 mM deoxy-NTPs, 2.5 μM random 9-mer primer, 20 U RNase inhibitor, and 2 μg total RNA, was incubated at 55 C for 60 min. RT was terminated by heating to 99 C for 5 min. Oligonucleotide primers for rat PDI for PCR were designed as previously reported (30). The forward and reverse primers for the 5′ upstream fragment of PDI were 5′-GGG GGG ATC CTC CGA CAT GCT GAG CCG TGC-3′ and 5′-AGC GAT GAC GAT ATT CTC AT-3′, respectively; the forward and reverse primers for the 3′ downstream fragment of PDI were 5′-ACC TGA TGA GCC AGG AAC TG-3′ and 5′-CCC TCG AGA GAT CTG GCT TCT GCA CTA C-3′, respectively. PCR was carried out for 35 cycles using Pyrobest DNA polymerase (Takara Bio, Inc.) as follows: denaturation at 96 C for 60 sec, annealing at 56 C for 60 sec, and extension at 72 C for 150 sec. After digestion with *Bam*HI and *Eco*RI, the 5′ upstream fragment of PDI and pBluescript vector (Stratagene, La Jolla, CA) were ligated. Subsequently, the 3′ downstream fragment of PDI and pBluescript vector containing the 5′ upstream fragment of PDI were digested with *Eco*RI and *Xho*I, then ligated. The resulting full-length rat PDI cDNA in pBlue-

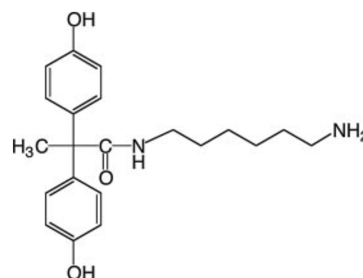

FIG. 1. Chemical structure of BPA amine derivative.

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script was subcloned. Next, the pBluescript vector containing the full-length rat PDI cDNA was digested with *Sac*I and *Kpn*I, then cloned into the histidine-tagged expression vector pQE-80L (QIAGEN, Valencia, CA). The resulting plasmid was transfected into *Escherichia coli* DH5α (Toyobo Co. Ltd., Osaka, Japan).

Purification of histidine-tagged fusion PDI

E. coli cells transformed with pQE-80L encoding the histidine-tagged PDI were grown at 37 C in 2× yeast extract-Tryptone rich medium containing 0.1 mg/ml ampicillin. Protein expression was induced by adding 1.0 mM isopropylthio-β-*O*-galactoside. After incubation for 4 h, *E. coli* cell were harvested and lysed in a lysis buffer (50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, 1.0 mg/ml lysozyme, and 0.5% protease inhibitor cocktail) for 60 min at 4 C. *E. coli* cell lysate was sonicated for 5 min, then incubated with 0.5% sucrose monolaurate for 60 min at 4 C with gentle stirring. The lysate sample was centrifuged at 50,000 × *g* for 30 min, and the supernatant was loaded onto a nickel-chelate-nitrilotriacetic acid agarose column (QIAGEN). After the column was washed with washing buffer (lysis buffer including 0.1% sucrose monolaurate), the protein was eluted with 50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl, 250 mM imidazole, 0.5% protease inhibitor cocktail, and 0.1% sucrose monolaurate. The eluted fraction was dialyzed against 50 mM Tris-HCl buffer (pH 7.5).

PDI-mediated isomerase activity

The oxidative renaturation activity mediated by PDI was measured according to the method described by Lyles *et al.* (31) with some modifications. Reduced and denatured RNase A (8 μM) was incubated with 1.4 μM PDI in a final volume of 0.5 ml 100 mM Tris-HCl buffer (pH 8.0) containing 4.5 mM cytidine 2′,3′-cyclic monophosphate, 2 mM EDTA, 1 mM glutathione, and 0.2 mM glutathione disulfide at 25 C. The reaction was started by adding reduced and denatured RNase A. The changes in absorbance at 296 nm were monitored. Reduced and denatured RNase A was prepared as follows. RNase A (type III) from bovine pancreas was incubated for 16 h in denaturing buffer [100 mM Tris-HCl buffer (pH 8.0) containing 140 mM dithiothreitol, 2 mM EDTA, and 6 M guanidine HCl] at room temperature. Denaturing buffer was exchanged for 0.1% acetic acid using a Bio-Gel P6 spin column (Bio-Rad Laboratories, Hercules, CA). The concentrations of reduced and denatured RNase A were calculated by absorbance at 280 nm using an extinction coefficient of 9300 cm^{-1} M^{-1}.

Others

NH_2-terminal amino acid sequences of the proteins were directly analyzed by automated Edman degradation using a protein sequencer (model 491, Procise, Applied Biosystems, Foster City, CA), after proteins were electrophoretically transferred to a polyvinylidene difluoride membrane at 2.0 mA/cm^2 membrane for 90 min in 100 mM Tris-HCl buffer (pH 8.3) containing 192 mM glycine and 20% methanol. Anti-PDI antiserum was prepared from rabbits immunized with purified histidine-tagged rat PDI. Anti-PDI IgG was purified using protein A-Sepharose CL-4B (Amersham Biosciences). SDS-PAGE and Western blot analyses were performed as described previously (32). The protein bands on SDS-PAGE gels were stained using a silver stain kit (Bio-Rad Laboratories, Inc.). For Western blot analysis, the nitrocellulose membrane was incubated with anti-PDI IgG for 1 h at room temperature. After washing three times with PBS containing 0.05% Tween 20 for 7 min each time, the nitrocellulose membrane was treated with the Vectastain ELITE ABC kit (Vector Laboratories, Inc., Burlingame, CA). Protein concentration was measured using the Bio-Rad protein assay kit. Kinetic analysis was performed using PRISM 3 (GraphPad, Inc., San Diego, CA). Animal treatments were performed under the standard methods of humane animal care. The protocol for this study was approved by the committee on the animal care and use of Osaka City University Medical School.

Results

BPA-binding proteins in rat brains

To explore target proteins of BPA, the BPA-binding activity of subcellular fractions (P2, P3, or S3) prepared from rat brains was determined (Fig. 2A). Each subcellular fraction of rat brains had specific BPA-binding activity, and the P2 fraction showed the highest binding activity, followed by the S3 fraction. To characterize BPA-binding activity in the rat brain P2 fraction, saturation experiments were performed with nine different concentrations of [^{3}H]BPA ranging from 3.05–52.5 μM. The data were analyzed by means of Scatchard plots, and the apparent dissociation constant (K_d), the maximal binding capacity (B_{max}), and the Hill coefficient were

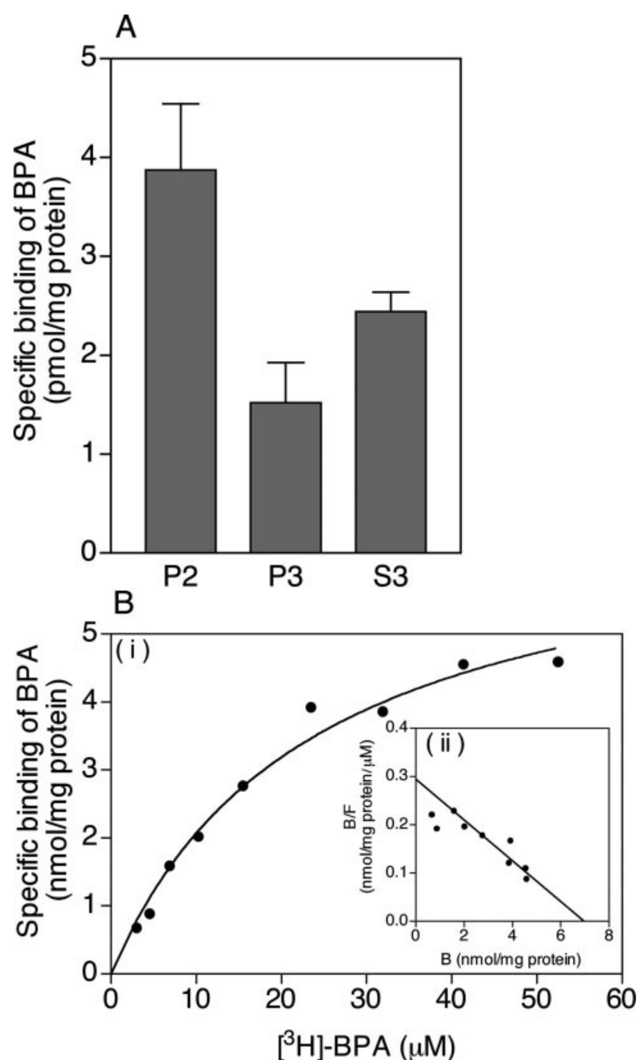

FIG. 2. BPA-binding activity in rat brains. A, BPA-binding activity of subcellular fractions prepared from rat brains. Each subcellular fraction (P2, P3, and S3 fractions; 1.5 mg protein) prepared from rat brains was incubated with 500 nM [^{3}H]BPA in 3.0 ml binding buffer [50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl] for 2 h at 4 C. Nonspecific binding was defined in the presence of 1 mM unlabeled BPA. Data shown are the mean ± SEM of triplicate determinations. B, BPA-binding activity of rat brain P2 fraction. P2 fraction (50 μg protein) was incubated with nine concentrations of [^{3}H]BPA, ranging from 3.05–52.52 μM in 0.5 ml binding buffer [50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl] for 2 h at 4 C. Nonspecific binding was defined in the presence of 1 mM unlabeled BPA. The data shown are the means of duplicate determinations. Kinetic values were calculated from three independent experiments, i, Saturation curve of BPA binding to rat brain P2 fraction; ii, Scatchard analysis of [^{3}H]BPA binding to rat brain P2 fraction.

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determined (Fig. 2B). Scatchard plots of the saturation curves were linear. The Hill coefficient was 1.07, indicating that BPA bound to one site or more with the same apparent affinity. The K_d of the rat brain P2 fraction for [^{3}H]BPA binding was 27.0 ± 3.9 μM, and the B_{max} was 7.4 ± 0.4 nmol/mg protein.

Purification of BPA-binding proteins

BPA-binding proteins were purified from rat brain P2 membrane fractions. Initially six detergents, Triton X-100, sodium dodecyl sulfate, sodium cholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Emulgen 913 (Kao Chemicals, Osaka, Japan), and sucrose monolaurate were tested to solubilize membrane proteins, and sucrose monolaurate was finally selected because of its high yield of solubilization and retention of BPA-binding activity (data not shown). The BPA-binding activity and protein concentration in each eluted fraction from Whatman DE52 column were measured (Table 1). The fraction eluted with 50 mM Tris-HCl buffer (pH 7.0) containing 0.2 M NaCl had the highest BPA-binding activity. The purified fraction eluted from BPA-Sepharose affinity resin showed a single protein band on SDS-PAGE analysis (Fig. 3A, lane 5). The molecular mass of this protein was calculated to be 53 kDa on an SDS-PAGE gel. The NH_2-terminal amino acid sequence of this purified protein was 1-DALEEEDNVLV-LKKSNFAEALAAHNYLLVEFYA-33, which is identical with that of rat PDI (29). In Western blotting analysis, the purified protein was recognized by anti-PDI antibody (Fig. 3B), confirming its identity as PDI.

BPA-binding properties of recombinant PDI

The BPA-binding activity of PDI was also characterized using recombinant rat PDI protein (rrPDI). For this experiment, first we cloned rat PDI cDNA, then expressed it in *E. coli* and purified the recombinant protein as a histidine-tagged fusion protein. Saturation experiments of BPA binding to rrPDI were performed with nine different concentrations of [^{3}H]BPA ranging from 0.4–43.1 μM. The data were analyzed by means of Scatchard plots, and the K_d, B_{max}, and Hill coefficient were determined (Fig. 4A). Scatchard plots were linear, and the Hill coefficient was 1.01. The K_d of rrPDI for [^{3}H]BPA was 22.6 ± 6.6 μM, and the B_{max} was 2.5 ± 0.8 nmol/mg protein. These kinetic parameters of rrPDI for

TABLE 1. The BPA-binding activity and protein contents in the fractions eluted from anion ion exchange chromatography

Fraction eluted by (M NaCl)	Total protein (mg)	Total BPA binding [dpm (×1000)]	BPA binding/protein [dpm (×1000)/mg]
0.1	15.3	50	3.3
0.2	5.0	135	27.0
0.5	12.0	140	11.7

Solubilized P2 membrane fraction was loaded onto a Whatman DE52 column (2.5 × 5.0 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0). Proteins were eluted with 12.5 ml of 50 MM Tris-HCl buffer (pH 7.0) containing 0.1, 0.2, or 0.5 M NaCl. For measurement of BPA-binding activity, 125 μl of each fraction was incubated with 500 nM [^{3}H]BPA in 3.0 ml of 50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl for 2 h at 4 C. The reaction mixture was aspirated rapidly through a GF/B filer, and the filter was washed three times with 3 ml ice-cold binding buffer. Protein concentration was measured by the method of Bradford. Data shown are means of triplicate.

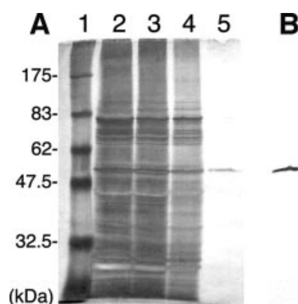

FIG. 3. SDS-PAGE and Western blotting analysis of the purified BPA-binding protein. A, SDS-PAGE analysis. The fractions in purification procedures were applied to a 10% SDS-PAGE gel. The protein bands were visualized by silver staining. Lane 1, Molecular mass standards; lane 2, the fraction eluted by 0.2 M NaCl from Whatman DE52 anion ion exchange chromatography (10 μl; 4 μg); lane 3, flow-through fraction from BPA affinity chromatography (10 μl; 10 μg); lane 4, washing fraction from BPA affinity chromatography (10 μl); lane 5, the fraction eluted by 2 mM BPA from BPA affinity chromatography (10 μl; 0.3 μg). B, Western blotting. The fraction eluted (10 μl; 0.3 μg) with 2 mM BPA from BPA affinity chromatography was applied to a 10% SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with anti-PDI IgG for 1 h at room temperature.

BPA-binding activity were comparable to those of the rat brain P2 fraction shown in Fig. 2B. These results confirmed that PDI possessed BPA-binding activity and also that the BPA-binding protein found in the rat brain P2 fraction was indeed PDI. In addition, our preliminary experiments using recombinant human PDI, the K_d value of BPA to recombinant human PDI was 17.51 ± 3.93 μM, a value close to what was observed with rrPDI. Based on these similar binding profiles, it is tempting to speculate that human PDI would also be sensitive to BPA-mediated effects.

PDI has been characterized as a multifunctional protein and shown to bind peptides, proteins, and hormones (33–36). A recent report suggested that there are at least three binding sites on the PDI molecule (34), with one site involved in protein/peptide binding, whereas the remaining two sites are hormone-binding sites. To determine which site(s) on PDI was responsive to BPA binding, competitive binding experiments were performed using unlabeled BPA, T_3, E_2, bacitracin, and InB as competitors (Fig. 4B). Both bacitracin, a cyclic peptide antibiotic, and InB are considered to be specific ligands for protein/peptide binding site on PDI (34). [^{3}H]BPA binding to rrPDI was competitively inhibited by both T_3 and E_2, but not by InB and bacitracin, to less than 20% of the total binding. This observation suggested that BPA binds to hormone-binding sites on PDI in a similar fashion to T_3 and E_2. Consistent with this idea, the IC_{50} values of E_2, T_3, and BPA for [^{3}H]BPA binding to rrPDI were 7.4, 8.0, and 19.6 μM, respectively.

Effects of BPA on T_3 and E_2 binding to PDI

To assess the effects of BPA on T_3 and E_2 binding to PDI, competitive binding experiments were performed. rrPDI was incubated with 100 nM [^{125}I]T_3 (Fig. 5A) or [^{3}H]E_2 (Fig. 5B) in the presence of competing ligands. It has been reported previously that the K_d value of T_3-binding activity to the high affinity site on PDI was 57 or 21 nM (35, 36). However, we did

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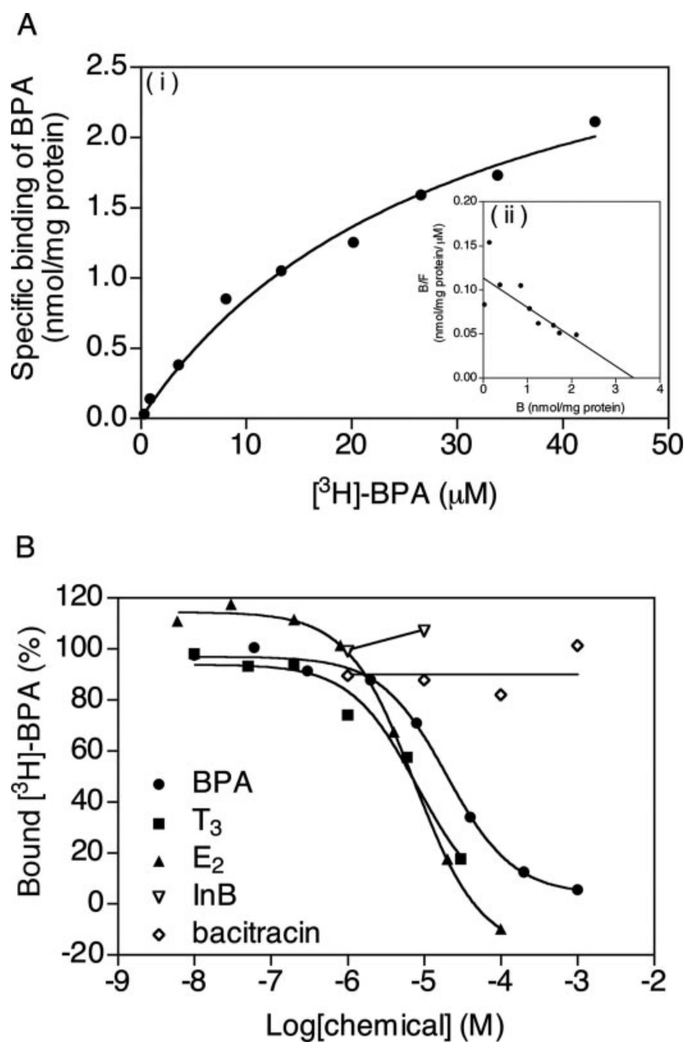

FIG. 4. A, BPA-binding activity of rrPDI. rrPDI (50 μg protein) was incubated with nine concentrations of [^{3}H]BPA ranging from 0.36–43.07 μM in 0.5 ml binding buffer [50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl] for 2 h at 4 C. Nonspecific binding was determined in the presence of 1 mM unlabeled BPA. Data shown are the means of duplicate determinations. Kinetic values were calculated from three independent experiments. i, Saturation curve of BPA binding to rrPDI. ii, Scatchard analysis of [^{3}H]BPA binding to rrPDI. B, Competitive BPA binding to rrPDI. rrPDI (50 μg protein) was incubated with 100 nM [^{3}H]BPA in the presence of competitors in 0.5 ml binding buffer [50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl] for 2 h at 4 C. T_3, E_2, InB, bacitracin, and unlabeled BPA were used as competitors in the assay. The data shown are the means of duplicate determinations and are expressed as a percentage of the total BPA binding.

not see such high affinity binding of T_3 to PDI in our experiments. Nonetheless, our data were comparable to those reported by Primm *et al.* (34). [^{125}I]T_3 binding activity to rrPDI was inhibited by both BPA and E_2 (Fig. 5A). The IC_{50} values of E_2, T_3, and BPA for [^{125}I]T_3 binding to rrPDI were 3.5, 1.6, and 38.0 μM, respectively. Also, [^{3}H]E_2 binding activity to rrPDI was inhibited by both BPA and T_3 (Fig. 5B). The IC_{50} values of E_2, T_3, and BPA for [^{3}H]E_2 binding to rrPDI were 2.5, 6.4, and 27.0 μM, respectively. These results suggested that BPA was able to work as a competitive inhibitor in PDI binding to T_3 and E_2, although the affinity of BPA to the

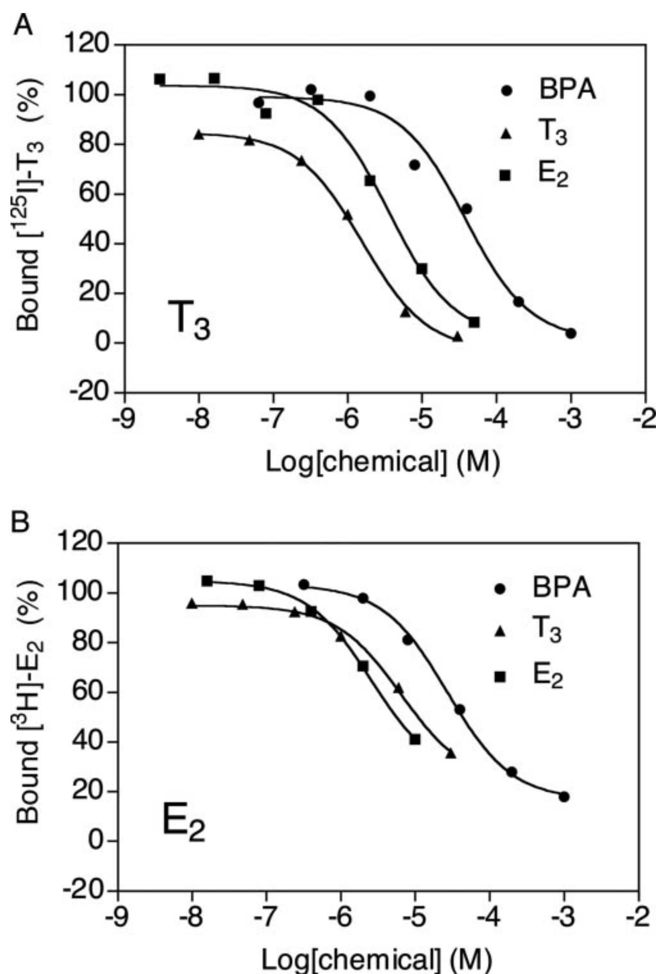

FIG. 5. Competitive binding assay of T_3 and E_2 to rPDI. rrPDI (50 μg protein) was incubated with 100 nM [^{125} I]T_3 (A) or [^{3}H]E_2 (B) in the presence of competitors in 0.5 ml binding buffer [50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl] for 2 h at 4 C. T_3, E_2, and unlabeled BPA were used as competitors. The data shown are the means of duplicate determinations and are expressed as a percentage of the total T_3 or E_2 binding.

hormone-binding site on PDI was lower than those of T_3 and E_2.

Effects of BPA on PDI-mediated isomerase activity

PDI is also known to catalyze disulfide formation and rearrangement by thiol/disulfide exchange in eukaryotes (33, 37). Primm *et al.* (34) previously reported that E_2 and T_3 did not inhibit the catalytic or chaperone activity of PDI. However, contrary to this view, others have reported that T_3 and E_2 can, in fact, inhibit PDI-mediated isomerase activity (36, 38). We assessed the effects of BPA on PDI-mediated isomerase activity (Fig. 6). BPA had an inhibitory effect on PDI-mediated isomerase activity. The percentage of maximum inhibition by BPA was calculated to be 24%, a value slightly less than that of T_3 or E_2. The IC_{50} of BPA was 3.72 μM, a value comparable to those of T_3 and E_2 (3.49 and 5.26 μM, respectively), indicating that BPA could work as a potential inhibitor of PDI activity similar to T_3 and E_2.

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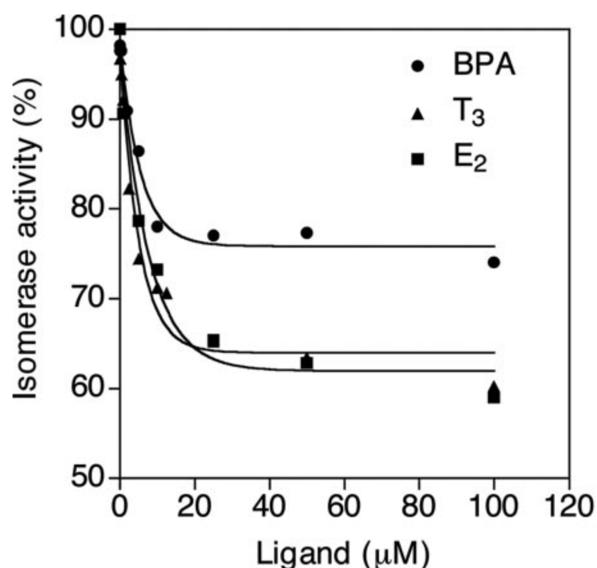

Kinetic parameters of BPA, T_3, and E_2 in isomerase activity of PDI

Ligand	max of inhibition (%)	IC_{50} (μM)
T_3	36	3.49
E_2	38	5.26
BPA	24	3.72

FIG. 6. Effects of BPA on PDI-mediated isomerase activity. The activity of PDI-mediated RNase refolding was determined in the presence of BPA, T_3, and E_2. Denatured RNase A (8 μM) was incubated with 1.4 μM PDI in a final volume of 0.5 ml 100 mM Tris-HCl buffer (pH 8.0) containing 4.5 mM cytidine 2′,3′-cyclic monophosphate, 2 mM EDTA, 1 mM glutathione, and 0.2 mM glutathione disulfide at 25 C. The data shown are the means of duplicate determinations and are expressed as a percentage of the total isomerase activity in the absence of ligands.

Discussion

We conducted the present study to identify target molecules of BPA and to delineate the mechanistic actions of BPA. We identified PDI as a target protein of BPA and demonstrated that its activities were inhibited by BPA. Although PDI was initially thought to be an endoplasmic reticulum-associated isomerase involved in the formation and/or reshuffling of the disulfide bridges in proteins, it is ubiquitously present in many organelles such as endoplasmic reticulum, nuclear envelope, and plasma membranes as well as in the cytosol of cells (33, 37, 39, 40). In our BPA binding experiment, although the rat brain P2 membrane fraction showed the highest BPA-binding activity, both the P3 membrane fraction and cytosol fraction also displayed some binding activity. It is plausible that this result might reflect the ubiquitous existence of PDI protein or, alternatively, the presence of other molecules with putative BPA-binding activity as has been described previously, such as nuclear hormone receptors, transthyretin, and CYP17 (23–29).

PDI is recognized to be a multifunctional protein, and its expression has been shown to be regulated by T_3 and E_2 (41–44). The rearrangement of disulfide bonds mediated by PDI is essential for cell viability in eukaryotes. PDI covalently binds to peptides and proteins and works as an isomerase (45). It also binds noncovalently to unfolded proteins to prevent their aggregation as a chaperone or to facilitate their aggregation as an antichaperone under certain conditions (46, 47). PDI-mediated isomerase activity is involved in the synthesis and regulation of many cellular proteins by mediating rearrangement of disulfide bonds (33, 37). For example, in an *in vitro* study, PDI was shown to be responsible for multimerization of thyroglobulin, which is a precursor of thyroid hormone, in the follicular lumen of the thyroid gland (48, 49). The membrane-associated PDI was also shown to participate in the reduction of disulfide bridges, resulting in the shedding of α-subunits of TSH receptors expressed on the plasma membranes of thyrocytes (50). PDI also has been implicated in the regulation of leukocytes adhesion (51). Thus, PDI appears to influence a multitude of physiological functions. In this study we demonstrated that BPA possesses inhibitory effects on PDI-mediated isomerase activity, suggesting that BPA might have potential in disrupting various physiological functions by its inhibitory actions on PDI.

PDI is also known to be a hormone-binding protein (34–36). Primm *et al.* (34) demonstrated that there are two hormone-binding sites and one peptide/protein-binding site on PDI. T_3 binds to both hormone-biding sites on PDI with comparable affinity, but E_2 binds to only one of the hormone-biding sites. However, in our experiments with rrPDI, the Scatchard plots for [^{3}H]BPA binding were linear, and the Hill coefficient was 1.01, indicating that BPA binds to a single site on PDI or, alternatively, to multiple sites with the same apparent affinity. In addition, the binding of BPA to rrPDI was competitively inhibited to less than 20% of the total binding by T_3 or E_2. Conversely, T_3 binding to rrPDI was almost completely inhibited by BPA, T_3, or E_2, and E_2 binding was also inhibited by BPA, T_3, and E_2. Based on these results, we propose that PDI has either one binding site for T_3, E_2, and BPA or two binding sites with the same affinity for each of these three compounds, contrary to what was previously proposed by Primm *et al.* (34).

The demonstration that BPA had inhibitory effects on T_3 and E_2 binding to PDI implies that BPA could potentially displace T_3 and E_2 from PDI. Because T_3 and E_2 are pivotally involved in brain development (52, 53), the physiological significance of T_3 and E_2 binding to PDI is of great interest and needs to be clarified. The K_d values of T_3 and E_2 binding to rrPDI were reported to be 4.3 and 2.1 μM, respectively (34). The affinities of T_3 and E_2 for their specific nuclear receptors are known to be in the nanomolar range; thus, the affinities of T_3 and E_2 for PDI are lower than those for their cognate nuclear receptors. However, PDI was identified, using a photoaffinity labeling technique, as a major T_3-binding protein in plasma membranes (39). PDI is ubiquitously expressed in cells and is reported to be an abundant protein in mammalian cells, corresponding to 0.35–0.4% of the total cellular protein in mouse and rat liver cells (54). From these observations, Primm *et al.* (34) estimated that approximately 90% of the intercellular hormones would bind to PDI and proposed that PDI may function to preserve the homeostasis of hormone molecules as a reservoir and to buffer the hormone concen-

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tration in the cells to allow hormones to bind to their cognate receptors.

Furthermore, PDI is thought to participate in the inactivation of type 2 iodothyronine 5′-deiodinase, which is a key enzyme for converting T_4 to bioactive T_3 in the brain (55, 56) and also in the association of estrogen receptor with estrogen response element (57). All these findings collectively suggest that PDI is likely to influence T_3 and/or E_2 hormonal activities in multiple ways. Conversely, T_3 and E_2 could alter the expression levels of PDI (41–44) and inhibit its isomerase activity (36, 38), although the physiological significance/role of T_3 and/or E_2 in regulating PDI expression and its activity remain to be clarified. Thus, it is likely that PDI might be closely involved in T_3 and E_2 functional and/or regulatory systems.

In conclusion, we report that the ubiquitous and multifunctional protein PDI is a target of BPA. We also propose a potential molecular mechanism that is operational via PDI to mediate the adverse effects of BPA. Our findings will facilitate understanding of multiple influences of EDCs, including BPA, on health and development and on the utility of using PDI in screening methods for EDCs.

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K.O., S.I., and M.O. have nothing to declare. T.H. and Y.F. are inventors (Japanese patent 2002-246351), based partly on this work.

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