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Interaction between Bisphenol Derivatives and Protein Disulphide Isomerase (PDI) and Inhibition of PDI Functions: Requirement of Chemical Structure for Binding to PDI

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Bisphenol A (BPA) is an endocrine disrupting chemical and several biological effects have been reported. Previously, protein disulphide isomerase (PDI) was isolated as a target molecule of bisphenol A. In this study, to clarify the effects of BPA on PDI functions, we investigated the relationship between the chemical structure of BPA derivatives and the effects on PDI-mediated isomerase and chaperone activity. We also investigated the effects of changes in the isomerase domain of PDI on the binding of chemicals, using PDI mutants and oxidized or reduced PDI. Among six chemicals, only chemicals, which have a phenol group, can bind to PDI and these chemicals also have an inhibitory effect on PDI-mediated isomerase activity. Changes in the structure of the PDI isomerase domain did not affect chemical-binding activity. On the other hand, the chemicals used in this study have low effects on chaperone activity of PDI. Substitutions in Cys residues (Cys398 and Cys401) of the isomerase active site changed chaperone activity. The present study indicates that phenolic compounds specifically bind to PDI and inhibit isomerase activity. This study provides useful information to predict the biological effects of chemicals and structural studies of PDI containing the function of chemical binding.

Key words: bisphenol A, protein disulphide isomerase (PDI), dimethyl bisphenol A, chaperone activity, protein folding.

Abbreviations: ADH, alcohol dehydrogenase; BPA, Bisphenol A, 2,2-bis(4-hydroxyphenyl)-propane; BPE, Bisphenol E, 1,1-bis(4-hydroxyphenyl)ethane; BPF, Bisphenol F, 4,4-methylenebisphenol; Cumylphenol, 4-α-Cumylphenol; DMBPA, dimethylbisphenol A, 2,2-bis(4-methoxyphenyl)propane; HIF-1α, hypoxia-inducible factor-1α; mBBr, monobromobimane; PDI, protein disulphide isomerase; RNase A, ribonuclease A; T3, 3,3′,5-triiodo-L-thyronine.

Bisphenol A (BPA, 2,2′-bis(4-hydroxyphenyl) propane) is a monomer of epoxy resin and is contained in industrial chemical products such as food cans, dental composites, baby-feeding bottles and plastic waste samples (*1*). BPA is known as an endocrine-disrupting chemical and exhibits estrogen-like effects on animals (*2*). Furthermore, it has been found that prenatal and neonatal exposure to BPA in mice induces hyper-locomotion and its sensitization induced by methamphetamine (*3*). In addition, we recently found that BPA affects the embryonic development of *Xenopus laevis*. Exposure of *Xenopus* embryos to BPA at stage 10.5 induced malformation of the head region and small-eye phenotype (*4*, *5*). These findings suggest that exposure to BPA at an early developmental stage may cause neuronal toxicity during embryonic development. We isolated a BPA binding-protein from the synaptosome fraction of the rat brain, which was identical to protein disulphide isomerase (PDI) (*6*). PDI is a 55 kDa protein which catalyses the formation, reduction or rearrangement of disulphide bonds of nascent or mis-folded protein (*7*). PDI is composed of **a**, **b**, **b**′, **a**′ and **c** domains. Two active sites exist in **a** and **a**′ domains, each of which includes two cystein residues. Thiol groups of PDI facilitate thiol-disulphide exchange (*8*). Previously, we investigated the effect of BPA on PDI mediate isomerase activity, and found that BPA inhibits PDI-mediated isomerization of reduced RNase (*6*). On the other hand, PDI has other functions than as a thiol-disulphide catalyst. Firstly, PDI serve as molecular chaperone, and prevent non-productive folding aggregation (*9*). Chaperone activity of PDI is known to be affected by cyclophilin B and cyclosporine A *in vitro* (*10*), but effects of BPA derivatives on chaperone activity have not been studied. Secondly, PDI is a thyroid hormone (T3)-binding protein (*11*). We found that BPA competitively binds to PDI with T3, and inhibits the binding of T3 to PDI in rat pituitary tumor (GH3) cells (*12*). Moreover, we found that BPA affects T3-induced growth hormone (GH) release and the GH level is influenced by the expression level of PDI (*12*).

PDI has been reported to play important roles in embryonic development or function of the brain and central nervous systems (*13*). Dysfunctions of PDI are found in some neurodegenerative diseases. Uehara *et al*. (*14*) suggested that *S*-nitrosylation of PDI inhibits PDI catalytic activity of PDI and links protein mis-folding

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to neurodegeneration. PDI or PDI family proteins inhibit the accumulation of mis-folded proteins triggered by endoplasmic reticulum (ER) stress and induce neurodegenerative diseases such as amyotrophic lateral sclerosis, prion neurotoxicity and Alzheimer disease (*14–17*). Regarding embryonic development, Zhang *et al*. (*13*) reported that ER stress occurs during development of the central nervous systems in the mouse, and the several ER-resident stress-regulated chaperones, including PDI family proteins and calreticulin, were expressed at higher levels in the embryonic brain and retina, compared with adult tissues. Hoshijima *et al*. (*18*) found that PDI is expressed in the embryonic midline and is required for left/right asymmetry. Taken together with these findings, BPA may induce disorders of PDI functions to induce neuronal dysfunction.

In this study, in order to clarify the effects of BPA on PDI functions, we investigated the relationship between chemical structure of BPA derivatives and effects on PDI-mediated isomerase activity and chaperone activity. We also investigated the effects of changes in the PDI active centre on the binding of chemicals to PDI, using a PDI which was mutated isomerase active sites, and oxidized or reduced PDI.

MATERIALS AND METHODS

2,2-Bis(4-hydroxyphenyl)propane (bisphenol A), 1,1-bis(4-hydroxyphenyl)ethane (bisphenol E) and 4,4-methylenebisphenol (bisphenol F) were 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). Sucrose monolaurate was purchased from Dojindo Laboratories (Kumamoto, Japan). Diphenylpropane, 3,3′,5-triiodo-L-thyronine (T3), ribonuclease A (RNaseA) type III from bovine pancreas, monobromobimane and alcohol dehydrogenase were purchased from Sigma-Aldrich Corp. (St Louis, MO). 4-α-Cumylphenol was purchased from Tokyo Chemical Industry (Tokyo Japan). 2,2-Bis(4-methoxyphenyl)propane (DMBPA) was prepared as described previously (*3*). BPA and other chemicals were dissolved in ethanol or dimethylsulfoxide (DMSO) at 100 mM to make stock solutions, which were stored at −80°C. T3 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.

Cloning and Expression of rat PDI Mutants—rat PDI cDNA was cloned as described previously (*6*). Rat PDI has two domains (**a**, **a**′) for isomerase activity. We prepared three mutants; by changing Cys residues (at Cys54 and Cys57 in **a** domain, and Cys398 and Cys401 in **a**′ domain) to Ala residues: mutant of **a** and **a**′, designated **aa**′m; mutant of **a**,**a**m; mutant of **a**′,**a**′m. PDI mutant (**aa**′m) was constructed from four fragments (Fig. 1). Fragment 1 was amplified with two primers, forward primer 1: 5′-GGGGGGATCCTCCGACATGCTGAGCCGTGC-3′ and reverse primer 1: 5′-TGGGGCCAGTGCTTTGGCGTGGCCAGCCCATGGGG-3′ (underline, complementary sequence to forward primer 2, and double underline, exchanged nucleotide for Cys to Ala). Fragment 2 was amplified with forward primer 2: 5′-AAAGCACTGGCCCCAGAGTATGCCAAAGC-3′ (underline, complementary sequence to reverse primer 1) and reverse primer 2: 5′-TCAAGTATGCGCTGGTTGTCAGTG-3′. Fragment 3 was amplified with forward primer 3: 5′-CTTTAAGGGCAAGATCCTGTTCAC-3′ and reverse primer 3: 5′-CGGGGCTAGCTGCTTGGCGTGACCAGCCCAGGGAG-3′ (underline, complementary sequence to forward primer 4, and double underline, exchanged nucleotide for Cys to Ala). Fragment 4 was amplified with forward primer 4: 5′-AAGCAGCTAGCCCCGATTTGGGATAAA-3′ (underline, complementary sequence to reverse primer 3) and reverse primer 4: 5′-TTGCGGCCGCGGCGCCCAGATCTGGCTTCTGCA-3′. The four amplified fragments were purified from an electrophoresis with an agarose gels. The second PCRs were performed with two fragments (fragment 1 and 2, or fragment 3 and 4) and two primers (forward primer 1 and reverse primer 2, or forward primer 3 and reverse primer 4) and then, third PCR was performed with two fragments (fragment 1 + 2 and 3 + 4) and two primers (forward primer 1 and reverse primer 4). The amplified fragment was subcloned into pGEM-T Easy (Promega Corp., Madison, WI), and then the cDNA of mutant rat PDI in pGEM-T Easy was digested with SphI and SalI, and subcloned into pQE-82L vector (Qiagen, Valencia, CA). PDI mutants **a**m and, **a**′m were prepared as follows. The C-terminal region of rat PDI WT in pQE-80L was cut out with ClaI and SalI, and subcloned into the pQE-82L plasmid, which includes **aa**′m, and digested with ClaI and SalI. The digested C-terminal region of rat PDI **aa**′m was subcloned into the pQE-80L plasmid, which includes WT, and digested with ClaI and SalI. The resulting plasmid was transfected into *Escherichia coli* (*E.coli*) DH5α (Toyobo Co. Ltd., Osaka, Japan).

Purification of Histidine-tagged Rat Fusion PDI—Rosetta gami B (Novagen, Madison, WI) *E. coli* cells transformed with pQE-80L encoding histidine-tagged rat PDI were grown at 37°C in 2× yeast extract-tryptone-rich medium containing 0.1 mg/ml ampicillin, 0.034 mg/ml kanamycin, 0.02 mg/ml tetracycline and 0.015 mg/ml chloramphenicol. Protein expression was induced by adding 1.0 mM isopropylthio-β-*O*-galactoside. After additional cultivation for 8 h, *E. coli* cells were harvested and lysed in a lysis buffer [50 mM NaH_2PO_4 (pH 7.5), and

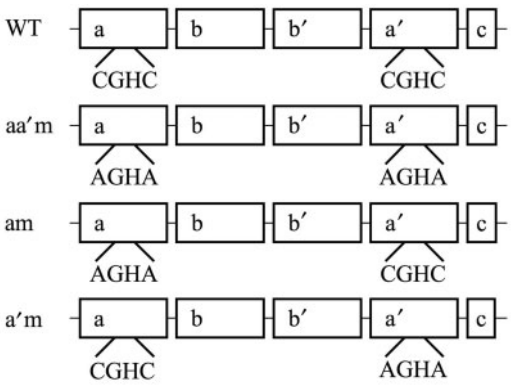

Fig. 1. **Domain structures of wild and mutant PDIs.**

300 mM NaCl], containing 1.0 mg/ml lysozyme and 20 mM imidazole for 60 min at 4°C. *E. coli* cell lysate was sonicated for 1 min, and then proteins were solubilized with 0.25% 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 Ni-NTA agarose column (Qiagen). After the column was washed with a buffer (lysis buffer including 0.1% sucrose monolaurate), the protein was eluted with lysis buffer containing 250 mM imidazole, and 0.1% sucrose monolaurate. The eluted fraction was dialysed against 50 mM Tris–HCl buffer (pH 7.5), and loaded onto an anion exchange column (MonoQ). Mono Q HR 10/10 column (Amersham Bioscience, Tokyo, Japan) was run with a linear gradient of 0.15-0.3 M NaCl in a 10 column volume of 20 mM Tris–HCl (pH 7.5) using an AKTA Explorer Systems (Amersham Bioscience).

Preparation of Oxidized PDI (Oxi PDI) or Reduced PDI (Red PDI)—Reduced PDI was prepared by incubating purified PDI (2 mg) with 10 mM DTT in a final volume of 1 ml of 50 mM MES (pH 6.3) for 18 h at room temperature. Separately, oxidized PDI was prepared by incubating 2 mg PDI with 2 mM GSSG (glutathione disulphide) in a final volume of 1 ml of 50 mM Tris–HCl (pH 7.5) for 18 h at room temperature, and then passed through Bio-Gel P6 spin column (Bio-Rad Laboratories, Hercules, CA) to remove DTT or GSSG.

Detection of Thiol Group by Monobromobimane (mBBr) Labelling and Fluorescence Detection—Red PDI and Oxi PDI (100 μl of each) were incubated with 20 mM mBBr (10 μl) at room temperature for 30 min. Excess mBBr was removed with a Bio-Gel P6 spin column, and then PDI was subjected to SDS–PAGE and visualized with UV.

Competitive Binding Assay to PDI—Purified histidine-tagged rat PDI was incubated with 100 nM [^{3}H]BPA in a 50 mM Tris–HCl buffer (pH 7.5), containing 150 mM NaCl for 120 min at 4°C with varying concentration of test chemicals in a final volume of 0.5 ml. Unbound [^{3}H]BPA was separated from bound [^{3}H]BPA according to the method of Horiuchi *et al*. (*19*) with some modifications. Briefly, after incubation, proteins were precipitated by adding equal volumes of 50 mM Tris–HCl buffer (pH 7.5), containing 12% polyethylene glycol 6,000, 150 mM NaCl and 200 mM $ZnCl_2$ to the reaction mixture, followed by centrifugation at 15,000*g* for 5 min at 4°C. The supernatant was aspirated, and then the pellet surfaces and insides of tubes were washed twice with 1.5 ml 50 mM Tris–HCl buffer (pH 7.5), containing 6% polyethylene glycol 6,000, 150 mM NaCl and 200 mM $ZnCl_2$. The pellets were dissolved with 0.1 N NaOH. Radioactivity was measured using a β counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA).

Isomerase Activity of PDI—Isomerase activity was assayed as described previously (*6*). Briefly, reduced and denatured RNase A (8 μM) was incubated with 1 μM PDI in 100 mM phosphate buffer (pH 8.0) containing 4.5 mM cytidine 2′,3′-cyclic monophosphate (cCMP), 2 mM EDTA, 1 mM GSH (glutathione) and 0.2 mM GSSG at 25°C in a final volume of 0.2 ml. The reaction was started by adding reduced and denatured RNase A. Changes in absorbance at 296 nm were monitored with a spectrophotometer, Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA).

Chaperone Activity of PDI—Thermally induced protein aggregation of ADH was measured as the turbidity of the solution monitored at 360 nm on a spectrophotometer at 40°C. Aggregation was initiated by the addition of 11 μM ADH to 50 mM HEPES–NaOH buffer (pH 7.0) containing 10 mM DTT, 1 μM PDI and test chemicals in a final volume of 200 μl. All aggregation experiments were normalized by control experiments, in which aggregation with the substrate alone was defined as being 100% of the total substrate aggregation.

RESULTS

The Required Structure of BPA Derivative Binding to PDI—To understand the relationships between the chemical structure of BPA derivatives and binding activity to PDI, competitive binding assay were performed using [^{3}H]BPA in the presence of BPA derivatives (Fig. 2). Cumylphenol inhibited [^{3}H]BPA binding to PDI as well as BPA, but diphenylpropane had low inhibitory effect (Fig. 3A), suggesting that the hydroxyl group of phenyl ring(s) is required for binding activity. Inhibition of [^{3}H]BPA binding to PDI by BPE and BPF was almost identical to BPA (Fig. 3B), suggesting that substitution of the methyl group at the centre of BPA did not contribute to the binding affinity to PDI. On the other hand, DMBPA did not inhibit [^{3}H]BPA binding to PDI, so the hydroxyl group of phenyl ring(s) is required for binding affinity.

Effects of Changes in the Structure of PDI Isomerase Domain on Binding of BPA Derivatives—The binding capacity of BPA to oxidized (Oxi) and reduced (Red) PDI was investigated. Using Walker's method (*20*), we prepared Oxi or Red PDI. Formation of a free thiol group was confirmed from bands which reacted with thiol-specific fluorescent probe mBBr. Values were normalized by the intensity of bands which detected by CBB staining (Fig. 4A). As a result, BPA binding activity of Oxi PDI or Red PDI was almost identical to WT PDI (Fig. 4B), and K_d values of BPA binding to Oxi and Red PDI were 8.49 and 16.87 μM, respectively (Fig. 4C). These results

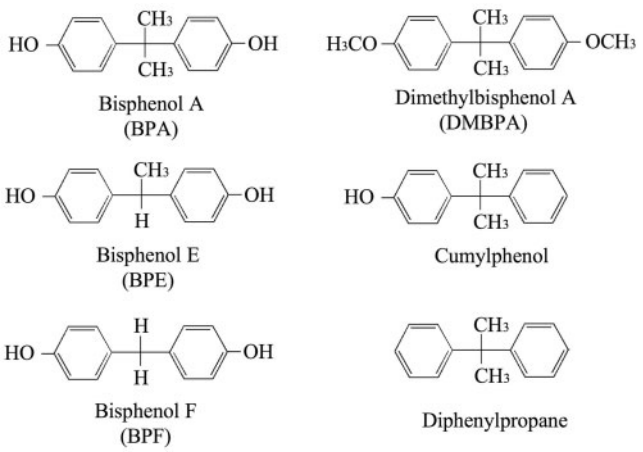

Fig. 2. **Structures of BPA derivatives.**

indicated that BPA binding activity was not change by the redox state of the isomerase active site. We next examined whether the Cys residue of the isomerase active site contributed to BPA binding activity. We purified three PDI mutants (Fig. 4D), and measured isomerase activity. As a result, BPA binding activity of all PDI mutants (**aa**′m, **a**m and **a**′m) was almost identical to WT PDI (Fig. 4D). These results indicated that the

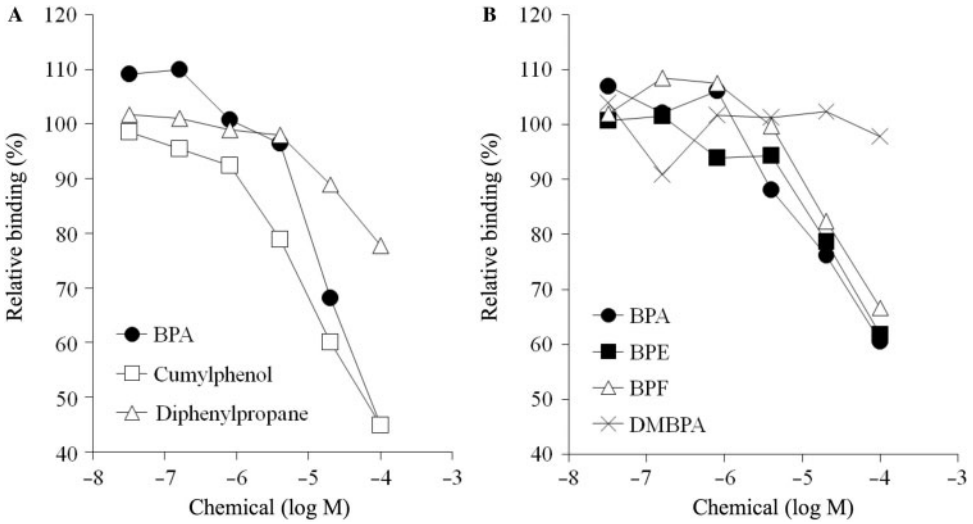

Fig. 3. **Competitive inhibition of [^{3}H]BPA binding by BPA derivatives.** 0.1 mg/ml of purified PDI was incubated with 100 nM [^{3}H]BPA in the presence or absence of test chemicals at various concentrations for 120 min at 4°C. Relative binding (%) is plotted against log concentration (M) of unlabeled test chemicals. Values with [^{3}H]BPA only were set at 100%. Data shown are the means of duplicate determining.

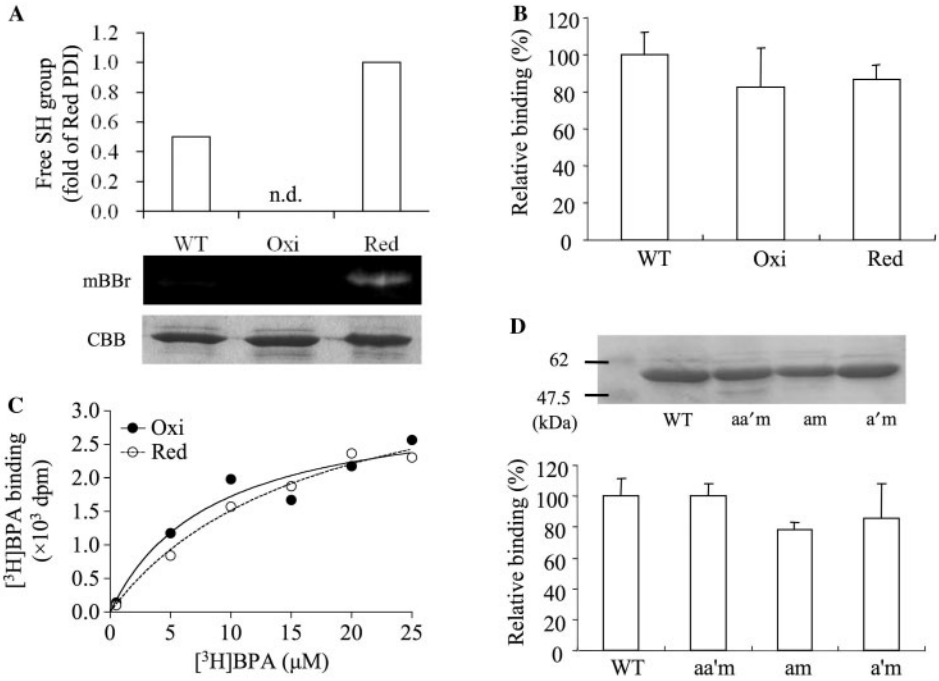

Fig. 4. **Binding assay for Oxi or Red PDI and PDI mutants.** The redox state of Oxi PDI and Red PDI were detected using mBBr (A). Red and Oxi PDI were treated with 2 mM mBBr. Labelling was carried out for 30 min at room temperature, excess mBBr was removed with Bio-Gel P6 spin column, and then samples were separated by SDS–PAGE and visualized with UV. The gel was subsequently stained with CBB. n.d. not detect. Binding activity of Oxi and Red PDI (B). 2 nM of Oxi and Red PDI were incubated with 100 nM [^{3}H]BPA for 120 min at 4°C. Values are the means ± SD of three replicates. Kinetic analysis of Oxi and Red PDI with BPA (C). PDI was incubated with six concentrations of [^{3}H]BPA. Non-specific binding was determined in the presence of 1 mM unlabeled BPA, and specific binding was calculated by subtracting the non-specific binding from total binding. Binding activity of PDI mutants (D). PDI mutants were constructed as described. Samples were separated by SDS-PAGE and detected by CBB staining. Refolding assay of reduced RNase A was performed in the presence of PDI mutants. 5nM of PDI mutants were incubated with 100nM [^{3}H]BPA for 120 min at 4°C. Values are the mean ± SD of three replicates.

Cys residue of the isomerase active site did not contribute to BPA binding activity.

Inhibition of Isomerase Activity of PDI by BPA Derivatives—To understand the relationships between the chemical structure of BPA derivatives and the inhibitory effect on PDI-mediated isomerase activity, isomerization of reduced RNase A to the active form was spectrophotometrically detected using cCMP as RNase substrate in the presence of BPA derivatives (Fig. 5). Each chemical was added to reaction buffer at a final concentration of 6.25, 12.5 and 25.0 μM. Both BPA and T3 effectively inhibited isomerase activity as previously reported (*6*). BPE and BPF also inhibited isomerase activity, suggesting that substitution of the methyl group at the centre of BPA did not contribute to the inhibitory effect. Cumylphenol inhibited isomerase activity, but diphenylpropane did not, suggesting that the hydroxyl group of the benzene ring is essential for the inhibitory effect of isomerase activity. These results indicated that inhibitory effect correlate with binding affinity to PDI.

Isomerase Activity of Oxi or Red PDI and PDI Mutants—We next performed an isomerase assay of Oxi PDI and Red PDI. GSSG promoted protein disulphide formation with PDI, and GSH promoted protein reduction to dithiol with PDI (*21*), so, to maintain the redox state of PDI, we performed an assay in the presence or absence of GSSG and GSH. Oxi PDI and Red PDI have similar activity for isomerization of reduced RNase A in spite of the absence of GSH and GSSG (Fig. 6A), suggesting that the redox state is not important for isomerase activity. We confirmed the contribution of each domain to isomerase activity. We found that **a**m and **aa**′m have defects in their of RNase A oxidation ability compared with WT PDI (Fig. 6B). In contrast, the activity of **a**′m was almost equal to WT PDI.

Inhibition of Chaperone Activity of PDI by BPA Derivatives—PDI also acts as a molecular chaperone. We examined the effect of BPA derivatives on chaperone activity of PDI. Thermal aggregation of ADH was detected by measuring the time-dependent change in turbidity (scattering) at 360 nm in the presence of test chemicals. Aggregation of ADH was suppressed by PDI (data not shown). The chemicals used in this study have low effects on chaperone activity of PDI (Fig. 7).

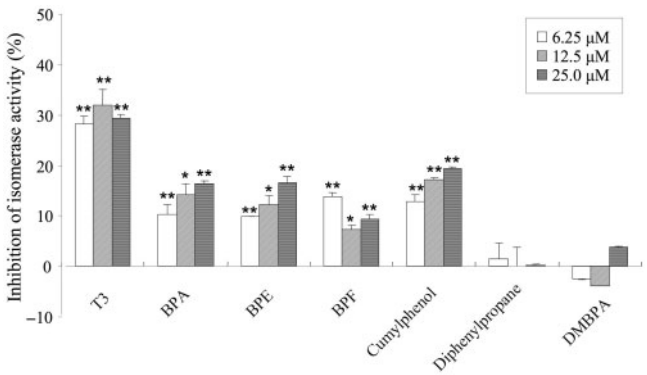

Fig. 5. **Effect of test chemicals on PDI-mediated isomerase activity.** Refolding assay of reduced RNase A was performed in the presence of test chemicals at various concentrations. The inhibition is relative shown to that observed in the absence of test chemicals as 0%. Values are the mean ± SD of three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

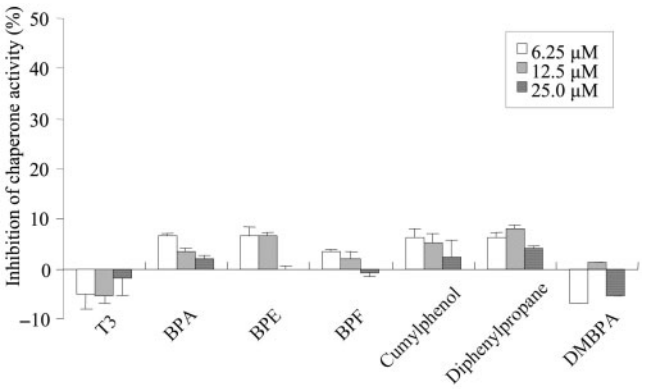

Fig. 7. **Effect of test chemicals on PDI-mediated chaperone activity.** Effect of test chemicals on chaperone activity of PDI. Native ADH was incubated in a spectrophotometer at 40°C in 50 mM HEPES, pH 7.0, with or without PDI and various concentration of chemicals. Thermal aggregation was monitored by measuring absorbance at 360 nm every minute. The rate of ADH aggregation (*ΔA*/min) was normalized to the rate observed in the absence of test chemicals as 0%. Values are the mean ± SD of three replicates.

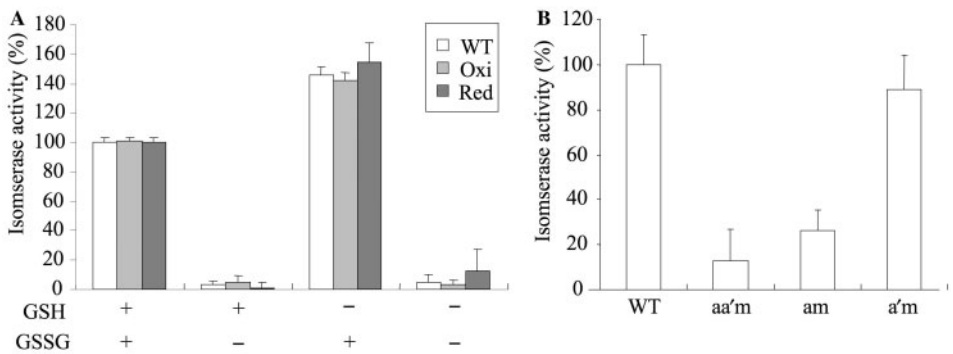

Fig. 6. **Isomerase activity of Oxi or Red PDI, and PDI mutants.** Refolding assay of reduced RNase A was performed in the presence of Oxi PDI or Red PDI (A) and PDI mutants (B). Isomerase activity of oxidized or reduced PDI was measured in the presence or absence of GSH or GSSG.

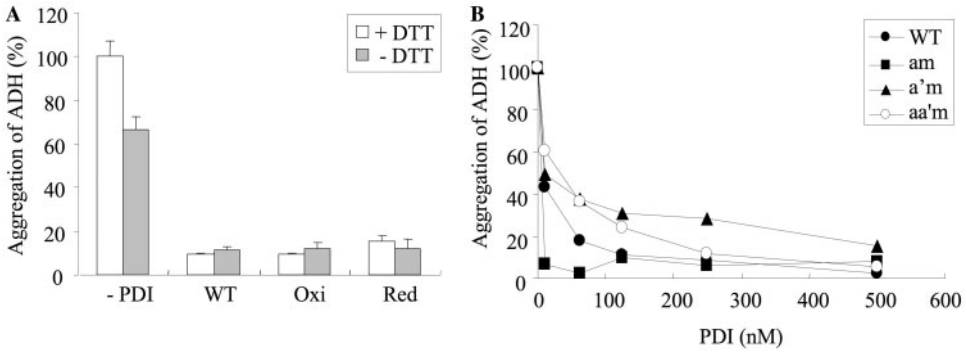

Fig. 8. **Chaperone activity of PDI mutants, and Oxi or Red PDI.** Native ADH was incubated in 50 mM HEPES pH 7.0, with various concentrations of PDI at 40°C. To determine the involvement of the redox state of PDI, chaperone activity of Oxi and Red was assessed in the presence or absence of DTT (A). The rate of ADH aggregation (ΔA/min) was normalized to the rate observed in the absence of PDI as 100%. Values are the mean ± SD of three replicates.

Contribution of PDI Isomerase Domain to Chaperone Activity—Next, to assess the contribution of the redox state of the active site to the chaperone activity, we determined chaperone activity of Oxi PDI and Red PDI. To eliminate the reduction of PDI by DTT, chaperone assay was performed in the absence of DTT, which was used as a promoter for aggregation of ADH. There were no significant differences between Oxi PDI and Red PDI in chaperone activity (Fig. 8A). These results suggest that the redox state of Cys residues of the PDI isomerase domain is not important for its chaperone activity. Moreover, to assess the contribution of Cys residues of active site to chaperone activity, we determined chaperone activities of PDI mutants. The mutant, **a**m, revealed higher chaperone activity than WT PDI, although chaperone activity of **a**′m and **aa**′m were lower than that by WT (Fig. 8B). These results strongly suggest that the PDI **a**′ domain plays an important role in the chaperone activity.

DISCUSSION

Previously, PDI was isolated from the rat brain as a BPA binding protein (*6*). In this study, to identify the basic PDI function involving the BPA binding activity, we performed experiments using six BPA derivatives, and oxidized or reduced PDI and three kinds of PDI mutants.

From the result of competitive binding assay, we found that the hydroxyl group of benzene ring(s) is required for binding affinity and substitution of centre the methyl group did not contribute to binding affinity. These results suggest that BPA derivatives bind to PDI via a hydroxyl group, and inhibit isomerase activity. BPA has been reported to interact with estrogen-related receptor gamma (ERRγ) via an important hydrogen bond between a phenol group of BPA and Arg316 of ERRγ, and a collaborative hydrogen bond between phenol group and Glu 275. We considered that BPA interacts with PDI via a hydrogen bond between the phenol group of BPA and hydrophilic amino acid of PDI as well as ERRγ (*22*, *23*).

ERRγ is identified as a high affinity nuclear receptor for BPA and its K_d value is 5.5 nM (*22*). Biological function of ERRγ is not well understood and effects of BPA on this receptor are not known. On the other hand, the K_d value of rat PDI for BPA was 22.6 μM (*6*) and much higher than that of ERRγ. However, PDI has been reported to be a high capacity and low affinity hormone reservoir of the endoplasmic reticulum (*11*). So, it is considered that BPA disrupts PDI functions in the cytosol or endoplasmic reticulum, but not in the nuclear. PDI expression is induced by unfolded protein response, and inhibits aggregation of protein by its isomerase or chaperone activity (*16*, *24*).

In order to investigate the relationship between the redox state of the isomerase active site of PDI and BPA binding activity, we performed a BPA binding assay using oxidized or reduced PDI. BPA binding activity was not changed by the redox state of PDI, suggesting that reduction or oxidation of isomerase active site does not change the structure of the chemical-binding site of PDI. Using a series of PDI mutants, we found that BPA binding activity was also not changed by substitution of Cys residues of isomerase active site, suggesting the Cys residues of the isomerase active site are not required for BPA binding activity. These results indicated that BPA does not bind to the isomerase active site of PDI.

In an isomerase assay with BPA derivatives, we found that the structural requirement for an inhibitory effect on PDI mediated isomerase activity is identical to the binding affinity to PDI. These results suggest that the inhibitory effect correlate with binding affinity to PDI. A previous report indicated that BPA degraded hypoxia inducible factor 1α (HIF-1α) via an unknown pathway and inhibited the hypoxia response, and that this phenomenon required two central methyl groups but not two phenol groups (*3*). However, in this study we found that central methyl groups in BPA did not contribute to the binding affinity to PDI and inhibitory effect on PDI-mediated isomerase activity, so the biological effect of BPA is not a simple pathway.

Next, to identify the contribution of the redox state of isomerase active sites or each Cys residue, we examined the isomerase activity of oxidized or reduced, and PDI mutants. We found that the redox state is not important for isomerase activity, while, from the experiments using three PDI mutants, we found that **a**m and **aa**′m have defective RNase A oxidation ability compared with WT PDI. Lyles *et al*. (*25*) reported that the isomerase active

site of **a**′ domain contributes more to apparent steady-state substrate binding, and the active site of **a** domain contributes more to catalysis at saturating concentration of substrate using human PDI. Because we used a lower concentration of PDI than Lyles's report, RNase A might saturate and the activity of **a** domain was more detectable than that of **a**′ domain.

PDI is also known as a molecular chaperone so we examined the effect of BPA derivatives on chaperone activity of PDI. As a result, any chemicals used in this study did not affect chaperone activity of PDI. We considered that ADH recognition by PDI was not affected by BPA and was different from RNase. We next investigated the participation of the redox state or mutation of isomerase active sites in chaperone activity. First, we found that redox state of isomerase active sites is not required for chaperone activity. On the other hand, chaperone activity was inhibited by the substitution of at least **a** domain. Whiteley *et al*. (*26*) indicated that the capacity for immunoglobulin solubility of **a**′m was lower than **a**m in the study using *Spodoptera frugiperda* (Sf-9) cells. Our result was reflecting *in vivo* situation. Puig *et al*. (*9*) reported that a 21 kDa C-terminal fragment of PDI also has chaperone activity; however, the chaperone activity of this fragment was lower than full-length of PDI. Taken together, the activity of **a** domain was not essential for chaperone activity, but was required for the facilitation of chaperone activity; therefore, chaperone activity is not dependent on isomerase activity.

Several reports indicate that accumulation of mis-folded protein by disfunction of PDI induce neurodegenerative diseases (*14*, *27*). *S*-nitrosylation of PDI inhibits catalytic activity of PDI and links protein mis-folding to neurodegeneration in Parkinson's or Alzheimer's disease (*14*, *27*). In addition, PDI is expressed in the embryonic midline and is required for left/right asymmetry (*18*). These findings suggest that PDI plays an important role in the function of brain and central nervous systems, and we can consider that BPA affects these functions of PDI and disrupts the formation of neuronal systems by accumulation of mis-folded proteins.

The present study indicates that phenolic compounds specifically bind to PDI and inhibit isomerase activity. Moreover, the inhibitory mechanism of isomerase activity was not by direct binding to isomerase active sites, so we must identify a BPA binding site in PDI. In fact, cumylphenol, BPE and BPF are also widely used as flame retardants in numerous products (*28*), so we are exposed to these chemicals in daily life. This study will provide useful information to predict the biological effects of chemicals. Moreover, this study also provides useful information to predict the biological effects of chemicals and structural studies of PDI with the function of BPA or T3 binding.

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