Repeated Exposure to 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), an Active Metabolite of Bisphenol A, Aggressively Stimulates Breast Cancer Cell Growth in an Estrogen Receptor β (ER β)–Dependent Manner

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ABSTRACT

Bisphenol A (BPA), recognized as an endocrine disruptor, is thought to exert its activity through a mechanism involving the activation of estrogen receptors (ERs) α/β . However, a major problem is that very high concentrations of BPA are required (i.e., those in excess of environmental levels) for effective activation of ER α/β -mediated transcriptional activities in vitro, despite the BPA-induced estrogenic effects observed in vivo. To elucidate the causal reasons, we successfully identified a BPA metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), which exhibits highly potent estrogenic activity both in vivo and in vitro. We have focused on the biologic relationship between breast tumor promotion and MBP/BPA, because BPA is considered to

be a human carcinogen owing to its breast tumor-promoting properties. In general, humans are exposed to many endocrine disruptors, including BPA. In the present study, we used the ER α/β -positive human breast cancer cell line MCF-7 as an experimental model to investigate the effects of repeated exposure to BPA/MBP at concentrations found in the environment on the expression of ER α/β and to determine the particular ER subtype involved. We demonstrated that repeated exposure to MBP, but not to BPA, significantly downregulated ER α protein expression and stimulated the proliferation of MCF-7 cells through the activation of ER β -mediated signaling.

Introduction

Ordinarily, humans are exposed to many chemicals present in the environment. Many of them, such as bisphenol A (BPA), can be used to produce polycarbonate and epoxy resins; these are used in a variety of consumer products, including baby bottles, food can lining, and dental sealant (Brotons et al., 1995; Olea et al., 1996; Yamamoto and Yasuhara, 1999). Thus, we may be exposed to BPA monomers that leach out from those materials. According to one study (Calafat et al., 2008), BPA has been detected in >90% of the human urine samples tested in the United States, which suggests the widespread nature of exposure to BPA. An accumulation of experimental evidence suggests the adverse health effects considered to be attributable to BPA include disorders of reproductive function, obesity, and cancer. In addition, there is reasonable evidence, such as breast/prostate tumor-promoting

properties, to suggest that BPA is a human carcinogen (Seachrist et al., 2016).

It is generally accepted that BPA is an endocrine disruptor that can exert estrogenic effects via estrogen receptors (ERs) α and β in vivo (Steinmetz et al., 1997; Welshons et al., 2006; Richter et al., 2007; Kundakovic et al., 2013), although it exhibits only very weak estrogenic properties in in vitro assays relative to 17 β -estradiol (E2), a physiologic ligand for ERs (Krishnan et al., 1993; Kuiper et al., 1997). To explain the inconsistency between the in vivo and the in vitro effects of BPA, it was proposed that BPA was susceptible to modification by certain drug-metabolizing enzyme-mediated metabolic processes that resulted in the formation of more active compounds in the in vivo environment. We originally identified 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), now recognized as an active metabolite of BPA, which can be obtained by incubation of BPA and liver S9 fraction, including human samples, in the presence of an NADPH-generating system. In the literature (Yoshihara et al., 2001, 2004), MBP was demonstrated to be a much stronger activator of ERs than is the parent compound BPA. Further, we found

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ABBREVIATIONS: ANOVA, analysis of variance; BPA, bisphenol A; CAS, Chemical Abstracts Service; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; FBS, fetal bovine serum; MBP, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene; MEM α , minimum essential medium α ; PC₁₀, transcriptional activities equivalent to 10% of the value of the positive control; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidin-3-yl]phenol; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction.

that the estrogenic activity of MBP was approximately 500-fold more potent than that of BPA in *in vivo* experiments performed in ovariectomized Wistar rats (Okuda et al., 2010). In addition, potent estrogenic effects were observed in experiments on medaka (*Oryzias latipes*) and zebrafish, in which MBP exhibited approximately 250-fold and 1000-fold stronger estrogenic activity than did BPA (Ishibashi et al., 2005; Moreman et al., 2018). As mentioned above, MBP is thought to be a key candidate for resolving the difference of *in vitro* and *in vivo* effects of BPA. We reported that MBP exhibited comparable EC₅₀ values for the activation of human ER α and ER β transiently expressed in NIH/3T3 cells (Yoshihara et al., 2004). Under physiologic conditions, the expression of ERs is regulated by their ligand, E2 (Nirmala and Thampan, 1995; Nawaz et al., 1999; Duong et al., 2006); this phenomenon suggested that prolonged, but not transient, exposure to MBP may lead to a change in the expression of ER α/β , which may enhance the malignancy of cancer cells.

Ligand-mediated ER degradation has been demonstrated in different cell types, including human breast cancer MCF-7 cells, which are known to be ER α/β positive (Nirmala and Thampan, 1995; Nawaz et al., 1999; Speirs et al., 1999a; Duong et al., 2006). The proliferation of MCF-7 cells is stimulated by E2, which results from the activation of the E2/ER α signal transduction pathways (Foster et al., 2001). Unlike ER α , the physiologic role of ER β is not fully understood; however, the β -type ER is recognized as a functional repressor of ER α owing to its ability to heterodimerize with ER α when they are coexpressed (Cowley et al., 1997; Ogawa et al., 1998; Pettersson et al., 2000; Powell and Xu, 2008). Furthermore, it has been suggested that ER β may be a tumor suppressor owing to its reduced expression during cancer development (Iwao et al., 2000). However, the accumulated evidence suggests that ER β also acts as a mediator of estrogen action in breast cancer cells (Speirs et al., 1999a; Hamilton et al., 2015; Ma et al., 2017). Thus, in the present study, we investigated the effects of repeated exposure to BPA, together with MBP, at concentrations similar to environmental levels on the expression of ER α/β , and investigated the ER subtype that was involved in these effects. Our results indicated that repeated exposure to MBP, but not BPA, downregulated ER α protein expression and stimulated the proliferation of MCF-7 cells through the activation of ER β .

Materials and Methods

Reagents. ICI 182,780 (fulvestrant) (purity, $\geq 98\%$) [Chemical Abstracts Service (CAS) number 129453-61-8] and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) (purity, $\geq 98\%$) (CAS number 805239-56-9) were purchased from Santa Cruz Biotechnology (Dallas, TX) and Sigma-Aldrich (St. Louis, MO), respectively. BPA [4,4'-(propane-2,2-diyl)diphenol] (purity, $\geq 99\%$) (CAS number 80-05-7) was purchased from Nacalai Tesque (Kyoto, Japan). The chemical synthesis of MBP (purity, $\geq 98\%$) (CAS number 13464-24-9) was performed in accordance with previously reported methods (Yoshihara et al., 2004; Okuda et al., 2010). E2 (purity, $\geq 98\%$) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Cell Cultures and Chemical Treatments. The cell culture conditions and methods used were based on previously described procedures (Takeda et al., 2013, 2016; Suzuki et al., 2017a). Briefly, the human breast cancer cell lines MCF-7 and MDA-MB-231 were routinely grown in phenol red-containing minimum essential medium

α (MEM α) (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10 mM HEPES, 5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μ g/ml), in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. Twenty-four hours prior to the treatment of cells with chemicals, the culture medium was changed to phenol red-free MEM α (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). ICI 182,780, PHTPP, BPA, and MBP were prepared in cell culture-grade dimethylsulfoxide. In the experiments in Fig. 1, B and C, the cells were treated with ICI 182,780 and PHTPP for 24 hours after cDNA transfection [see Transfection and Luciferase Analysis (Dual-Luciferase Assay)] and then collected for the dual-luciferase assay. The details of the chemical treatment method, except for the experiment of Fig. 1, are shown in Fig. 2B. The first treatment (treatment 1) comprised vehicle (for the control) or ligands [BPA or MBP, for the booster (–/+)]. After 24 hours of the first treatment, the culture medium was changed to fresh phenol red-free MEM α , and the cells were further treated with the vehicle [for control or booster (–)] or with ligands [for booster (+); treatment 2]. The results shown in Fig. 8 are from experiments performed after MBP treatment in the presence or absence of PHTPP. The results shown in Fig. 9 are from experiments in which the cells were treated with 2 nM MBP for 48 hours without alteration of the culture medium (indicated as 2 nM MBP), to compare the conditions between 2 nM MBP and booster (+) (see Fig. 9A). After a total treatment time of 48 hours, the cell samples were collected for the dual-luciferase assay, cell proliferation analysis, cell cycle analysis, Western blotting, and real-time reverse-transcription polymerase chain reaction (RT-PCR).

Transfection and Luciferase Analysis (Dual-Luciferase Assay). The experiments were performed as described previously, with minor modifications (Takeda et al., 2013; Okazaki et al., 2018). In brief, 24 hours prior to transfection, MCF-7 and MDA-MB-231 cells (5×10^4 cells/well) were seeded in 24-well plates containing MEM α . Each expression plasmid was transfected by using Lipofectamine LTX with PLUS reagent (Thermo Fisher Scientific, Waltham, MA). DNA mixtures containing 300 ng of estrogen-responsive element (ERE)₃-luciferase plasmid were cotransfected with 2 ng of *Renilla* luciferase reporter plasmid in 24-well plates. At 24 hours after transfection, the cells were washed with phosphate-buffered saline, the culture medium was exchanged for phenol red-free MEM α -supplemented with 5% dextran-coated charcoal-treated FBS, and chemical treatments were applied in accordance with the methods described in Cell Cultures and Chemical Treatments. After 24 or 48 hours of treatment, cell extracts were prepared in 100 μ l of passive lysis buffer (Promega, Madison, WI), from which 20 μ l was extracted to perform the firefly luciferase and *Renilla* luciferase assays (Dual-Luciferase Reporter Assay System; Promega). The ratio of firefly luciferase activity to *Renilla* luciferase activity was presented as the relative luciferase activity.

Cell Proliferation Analysis (MTS Assay). The MTS assay was performed as described previously (Takeda et al., 2016; Suzuki et al., 2017b). Briefly, the cells were seeded into 96-well plates at a density of $\sim 5 \times 10^3$ cells/well in the cell proliferation study. After chemical treatments or incubation, the degree of cell proliferation was analyzed by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS reagent; Promega).

Cell Cycle Analysis. The chemically treated cells were harvested (2×10^5 cells/sample) and fixed in 70% ethanol at 4°C overnight. The fixed cells were harvested and resuspended in 450 μ l of PBS containing 50 μ g/ml propidium iodide (PI) (Nacalai Tesque) and 100 μ g/ml RNaseA (Qiagen, Inc., Hilden, Germany) and incubated at 26°C for 30 minutes in the dark. Finally, the stained cells were analyzed by using a FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ). A minimum of 1×10^4 cells was counted per sample. The obtained results were analyzed by using ModFit LT version 3.0 (Verity Software House, Topsham, ME).

Fig. 1. ER β inhibition of transcriptional activity mediated by ER α in MCF-7 cells. (A) A model of ER α signaling abrogation by ER β . (B and C) MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, the cells were treated with ICI 182,780 (1 pM to 1 μ M) (B) or PHTPP (10 pM to 10 μ M) (C). The control sample was incubated with vehicle (indicated as Ctl.). After 24 hours, the cells were harvested and assayed for luciferase activity, with all transfection efficiencies normalized by using the internal *Renilla* control plasmid. The data are presented as the mean $\pm$ S.D. ($n = 3$) fold induction from the vehicle-treated control (indicated as 1). Significant differences [for (B and C) one-way ANOVA, followed by Dunnett's post hoc test] compared with control are marked with asterisks (* $P < 0.05$).

Antibodies and Western Blotting. Antibodies specific for ER α (ab79413; Abcam, Cambridge, MA), ER β (ab3576; Abcam), and β -actin (sc-47778; Santa Cruz Biotechnology) were used. Whole-cell extracts were prepared by using lysis buffer (50 mM HEPES-NaOH at pH 7.5, 25 mM NaCl, 1% NP40, and 1 mM phenylmethylsulfonyl fluoride) supplemented with cComplete Mini Protease Inhibitor Cocktail (Sigma-Aldrich). The cell extracts that were transfected with human ER α or ER β cDNA were used as the positive control (indicated as Std.; Figs. 6 and 9B). SDS-PAGE/Western immunoblotting was performed based on previously described procedures (Suzuki et al., 2017b). The antibodies for ER α (1:2000 dilution), ER β (1:2000 dilution), and β -actin (1:4000 dilution) were used as the primary antibodies for the detection of ER α , ER β , and β -actin, respectively. Anti-rabbit IgG (1:10,000 dilution) and anti-mouse IgG (1:10,000 dilution) antibodies conjugated with peroxidase were used as the secondary antibodies. Luminata Forte Western HRP Substrate (Millipore, Billerica, MA) was used for the detection of blotted bands. The quantification of band intensities was performed by using ImageJ 1.46r software (<http://imagej.nih.gov/ij/>), and the obtained values were normalized to β -actin, an endogenous control.

Preparation of Total RNA and Real-Time RT-PCR. Real-time RT-PCR was performed as described previously (Okazaki et al., 2018). Briefly, total RNA was prepared from MCF-7 cells by using TRIzol RNA Isolation Reagent (Thermo Fisher Scientific). cDNA was synthesized from 600 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time RT-PCR was

Fig. 2. Structures and experimental scheme for the treatment with BPA or MBP. (A) The structures of BPA and MBP. (B) The experimental scheme for treatment with BPA or MBP. In treatment (1), the cells were treated with vehicle for control or ligands (BPA or MBP) for booster (-) and booster (+). In treatment (2), the culture medium was changed after 24 hours of treatment (1), and the cells were then treated with vehicle for control/booster (-), or ligands for booster (+). The details of the experimental scheme for the treatments are described in the *Materials and Methods*. After chemical treatment for a total of 48 hours, samples of the treated cells were collected for the analysis of gene expression, luciferase activity, cell proliferation, and cell cycle.

performed using an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific) with Fast SYBR Green Master Mix (Thermo Fisher Scientific). The following primers used: ER α (sense), 3'-GAA AGG TGG GAT ACG AAA AGA CC-5'; ER α (antisense), 3'-GCT GTT CTT CTT AGA GCG TTT GA-5'; 18S-ribosomal RNA (sense), 3'-CGG CTA CCA CAT CCA AGG A-5'; and 18S-ribosomal RNA (antisense), 3'-GCT GGA ATT ACC GCG GCT-5'. The mRNA expression of ER α in each sample was normalized to the corresponding mRNA expression of 18S-ribosomal RNA.

Data Analysis. The EC₅₀ values of BPA and MBP were determined, and the concentrations of the compounds that elicited ERE-mediated transcriptional activities equivalent to 10% of the value of the positive control (1 nM E2) were defined as PC₁₀ (EPA, 2011). Dose-response curves were fitted by using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA). Differences were considered significant for P values less than 0.05. The statistical significance of differences between the two groups was calculated by using the Student's t test. Other statistical analyses, such as Dunnett's or Tukey-Kramer tests, were performed as post hoc tests after the analysis of variance (ANOVA) (details are indicated in figure legends). These calculations were performed by using StatView 5.0 J software (SAS Institute Inc., Cary, NC).

Results

ER β as a Negative Regulator of ER α -Mediated Transcriptional Activity. It has been reported that estrogen signaling can be modulated by several methods; for

Fig. 3. Effects of repeated exposure of BPA and MBP on the proliferation of MCF-7 cells. MCF-7 cells were exposed to BPA (0.1 nM to 10 μ M) (A) or MBP (0.1 nM to 10 μ M) (B), and then cell proliferation was measured. The chemical treatments and cell proliferation analysis were performed in accordance with the methods described in *Materials and Methods*. The control sample (indicated as Ctl.) comprised incubation with vehicle alone. The open and closed circles represent booster (-) and booster (+), respectively. The data are presented as the mean $\pm$ S.D. ($n = 6$) of percentage relative to the control. Significant differences [for (B) two-way ANOVA, repeated (related) measures with Tukey-Kramer's post hoc test] are marked with asterisks (* $P < 0.05$). P values were for the line graph obtained when MBP was treated at concentrations up to 25 nM, compared with the booster (-) line graph.

example, when ER α and ER β are simultaneously expressed in cells, E2/ER α -mediated transcriptional activity can be suppressed by the β -type ER in MCF-7 and other cell lines, including in human embryonic kidney 293 cells cotransfected with ER α /ER β expression plasmids (see the model described in Fig. 1A) (Pettersson et al., 2000; Takeda et al., 2013; Takeda, 2014). It is generally considered, especially in in vitro conditions, that the expression of the individual ERs is one of the key determinants for the output of E2 signaling. ER α -positive MCF-7 cells produce endogenous E2, but at very low levels, to stimulate their own growth via E2/ER α and express the ER β subtype (Miki et al., 2007). Therefore, in this study, we selected the MCF-7 cell line as a model and investigated the effects of two established antagonists, ICI 182,780 and PHTPP, which are specific for ER α and ER β , respectively, in the absence of exogenous E2. As shown in

Fig. 4. Repeated exposure of MBP stimulated cell cycle progression in MCF-7 cells. The MCF-7 cells were treated with 1 nM MBP for booster (-) and booster (+) and then stained with PI. The distribution of cells in each phase of the cell cycle was determined by flow cytometric analysis of PI staining of the DNA. (A) Representative histograms are shown. (B) The expected phase of the cell cycle and the percentage of cells in each phase are indicated. The data are presented as the mean $\pm$ S.D. ($n = 3$). Significant differences [for (B) two-way ANOVA, followed by Dunnett's post hoc test] compared with control are marked with asterisks (* $P < 0.05$).

Fig. 1, B and C, ER/ERE-mediated transcriptional activity in MCF-7 cells was inhibited by ICI 182,780 in a concentration-dependent manner up to 1 μ M, whereas PHTPP stimulated the transcriptional activity in a concentration-dependent manner up to 10 μ M. These data strongly suggested that E2/ER-mediated estrogen signaling was triggered mainly by the ER α subtype and that ER β exerted a negative impact on ER α in the conditions in which cells coexpressed both subtypes (Fig. 1A).

MBP as a Positive Stimulator of ER-Positive Breast Cancer Cell Proliferation in a Repeated Exposure-Dependent Manner. Humans are routinely exposed to a variety of environmental chemicals, including BPA, which can act as ligands and stimulate ERs. It is important to investigate whether repeated exposure of BPA to cells that express both ER subtypes may result in the perturbation of ER α /ER β expression, which may be coupled with the dysregulation of estrogen receptor signaling. Therefore, we first investigated

Fig. 5. Effects of repeated exposure of BPA and MBP on transcriptional activity mediated by ERs in ER α / β -positive MCF-7 cells. MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, the cells were treated with BPA (25 nM to 10 μ M) (A and B) or MBP (0.25 to 100 nM) (C and D) for booster (-) (left panel) and booster (+) (right panel). The control sample (indicated as Ctl.) was treated with vehicle. After chemical treatment for a total of 48 hours, the cells were harvested and assayed for luciferase activity; all transfection efficiencies were normalized by using the internal *Renilla* control plasmid. (A–D) EC_{50} and PC_{10} values were determined. Details are described in *Materials and Methods*. The data are presented as the mean $\pm$ S.D. ($n = 3$) of the fold induction from the vehicle-treated control (indicated as 1). N.D., not determined (because of low estrogenic activities not displaying a saturation curve profile).

the effects of BPA and its metabolite MBP (structures described in Fig. 2A) on the proliferation of MCF-7 cells. MCF-7 cells were treated with individual bisphenols in accordance with the experimental scheme described in Fig. 2B [i.e., booster (-) vs. booster (+)]. As shown in Fig. 3A, there were no observable effects of repeated exposure of BPA on the proliferation of MCF-7 cells, although BPA stimulated cell proliferation in a concentration-dependent manner up to 10 μ M. In contrast, when the effect of MBP was examined, cell proliferation was positively stimulated by the molecule in a repeated exposure-dependent manner up to 25 nM [i.e., booster (+)]; conversely, MCF-7 cell growth was suppressed by MBP concentrations above 2.5 μ M (Fig. 3B). To conduct further study of whether the MBP-mediated “booster effects” were dependent on the expression status of ERs in cells, we used the human breast cancer cell line MDA-MB-231, which is ER negative (Weigel and deConinck, 1993; Takeda et al., 2013). MBP did not exert a stimulatory effect on the proliferation of MDA-MB-231 cells (data not shown). To confirm the phenomenon observed in Fig. 3B, cell cycle analysis was performed by using flow cytometry. In support of the MBP-mediated upregulation of MCF-7 cell growth, the percentage of cells in the S phase (i.e., DNA replication), which is a preliminary step in cell division, was clearly increased in an MBP treatment-dependent manner [i.e., 25.4% for control, 36.9% for booster (-), and 45.7% for booster (+)] (Fig. 4, A and B). These results indicated that MBP may positively modulate

the proliferation of ER-positive MCF-7 cells through the mitogenic activity of cell cycle acceleration. Thus, questions arose on the nature of the driving force for the MBP-mediated stimulation of MCF-7 cell proliferation associated with the stimulation of cell cycle progression. To address this issue, we studied the effects of BPA and MBP on ER/ERE-mediated transcriptional activity in the presence or absence of repeated exposure (see Fig. 2B). The panels in Fig. 5 show the line graphs representing dose-response profiles for the estrogenic activities of BPA and MBP, together with the data of 1 nM E2, which was used as the positive control. As BPA and MBP displayed lower estrogenic potency in booster (-) than in booster (+) conditions, accurate EC_{50} values were not obtained (indicated as N.D.); thus, we also determined the value of PC_{10} . The comparison of the EC_{50} values for the activation of ERs by BPA and MBP revealed that remarkable boosting effects were observed after the treatment of both BPA and MBP (BPA: PC_{10} values of 665 nM [booster (-)] vs. 138 nM [booster (+)]; MBP: PC_{10} values of 4.8 nM [booster (-)] vs. 0.6 nM [booster (+)] (Fig. 5, A–D). In addition, when focusing on the EC_{50} values between BPA and MBP in booster (+) conditions, much lower EC_{50} values were found for MBP exposure (519 vs. 2.8 nM) (Fig. 5, B and D).

MBP Induced Downregulation of ER α mRNA/Protein in a Repeated Exposure-Dependent Manner. It has been reported that the ER α protein may be subject to ligand-mediated degradation, preferentially in the presence of E2 (i.e., >1 nM) (Nirmala and Thampan, 1995; Nawaz et al.,

Fig. 6. Effects of repeated exposure to BPA and MBP on the protein expression of ER α and ER β in MCF-7 cells. (A and B, top panel) Western blotting of ER α and ER β . MCF-7 cells were treated with 1 nM BPA (A) or MBP (B) for booster (-) and booster (+) or vehicle (control). After chemical treatment for a total of 48 hours, total cell lysates were prepared, and Western blotting was performed by using antibodies specific for ER α , ER β , and β -actin, respectively; β -actin was used as an internal loading control. The cell lysates prepared with human ER α or ER β cDNA-transfected cells were used as the positive control (indicated as Std.) (A and B, bottom panel). The band intensity of ER α (left panel) and ER β (right panel) was quantified by using ImageJ 1.46r software, and the values were normalized to those of β -actin. The data are presented as the mean $\pm$ S.D. ($n = 3$) of the fold change from the vehicle-treated control (indicated as 1). Significant differences (two-way ANOVA, followed by Dunnett's post hoc test) compared with control are marked with asterisks (* $P < 0.05$).

1999). Given that MBP behaves as an ER α ligand to induce similar biologic effects in E2, the protein expression of ER α may be downregulated after repeated exposure to MBP. Thus, we analyzed the protein expression of ER α , together with ER β , in MCF-7 cells. To use BPA concentrations that reflected those found in biologic samples (Schönfelder et al., 2002; Welshon et al., 2006; Vandenberg et al., 2010), we treated MCF-7 cells with or without repeated exposure of BPA and MBP at 1 nM. When the membranes were blotted for BPA, no modulatory effects of 1 nM BPA on ER α / β expression were

Fig. 7. Effects of repeated exposure of BPA, MBP, and BPA/MBP on the mRNA expression of ER α in MCF-7 cells. Real-time RT-PCR analysis of ER α . Booster (-) (A), booster (+) (B) MCF-7 cells were treated with 1 nM BPA, 1 nM MBP, or a combination (BPA/MBP) for booster (-) and booster (+) or vehicle (indicated as Ctl.) for 48 hours. The data are presented as the mean $\pm$ S.D. ($n = 6$) of the fold induction from the vehicle-treated control. Significant differences (two-way ANOVA, followed by Dunnett's post hoc test) compared with control are marked with asterisks (* $P < 0.05$).

found relative to the control in either condition (booster +/-); however, the BPA metabolite MBP did reduce the expression of ER α in a treatment-dependent manner without significant alteration of the protein expression of ER β , although there was a tendency for ER β to be upregulated (Fig. 6, A and B). To determine whether MBP also affected ER α mRNA expression after repeated exposure, we performed real-time RT-PCR analysis of samples derived from both conditions (booster +/-). Similarly, as shown in Fig. 7, 1 nM MBP, but not 1 nM BPA, downregulated the mRNA expression of ER α in a repeated exposure-dependent way. Collectively, these data suggested that the protein expression ER α can also be downmodulated by MBP via a reduction in ER α mRNA.

Involvement of ER β in MBP Estrogenic Action in a Repeated Exposure-Dependent Manner. We further investigated whether the MBP-mediated booster effects were dependent on the basal expression status of ERs (ER α and ER β); for this, the MDA-MB-231 cell line, which is ER α

Fig. 8. Effects of the ER β antagonist PHTPP on MBP-stimulated transcriptional activity and cell proliferation. (A) MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid. After transfection, the cells were treated with MBP (0.25–100 nM) for booster (+) in the absence (MBP only) or presence of 0.1, 0.25, 0.5, or 1 μ M PHTPP. The control sample was treated with vehicle (indicated as Ctl.). After chemical treatment of a total of 48 hours, the cells were harvested and assayed for luciferase activity; all transfection efficiencies were normalized by using the internal *Renilla* control plasmid. The data are presented as the mean $\pm$ S.D. ($n = 3$) of the fold induction from the vehicle-treated control (indicated as 1). (B) MCF-7 cells were exposed to 1 nM MBP for booster (+) in the absence or presence of 0.1, 0.25, 0.5, or 1 μ M PHTPP, and then cell proliferation was measured. A control incubation was treated with vehicle alone (indicated as Ctl.). The data are presented as the mean $\pm$ S.D. ($n = 6$) percentage of the control. Significant differences (two-way ANOVA, followed by Dunnett's post hoc test) compared with control are marked with asterisks (* $P < 0.05$).

negative and has a very low expression of ER β , was used (Weigel and deConinck, 1993; Takeda et al., 2013). As expected, it was clearly shown that neither the MBP-mediated stimulation of ER/ERE nor the MBP booster effect were detected in MDA-MB-231 cells (data not shown). To determine whether functional ER β was indeed involved in the MBP-induced transcriptional activation of ER β /ERE in MCF-7 cells after repeated exposure [i.e., booster (+) conditions], we performed an ERE-luciferase analysis of samples that were treated with MBP in combination with four different concentrations of PHTPP (i.e., 0.1, 0.25, 0.5, and 1 μ M), a selective antagonist of the ER β subtype. When compared with the MBP-only treatment group at concentrations between 0.25 and 100 nM, the MBP-activated transcriptional activities were clearly attenuated by PHTPP in a concentration-dependent manner (Fig. 8A). Furthermore, we analyzed

Fig. 9. Comparison of the effects of repeated exposure of 1 nM MBP [booster (+)] and 2 nM MBP exposure on ER α / β protein expression and cell proliferation in MCF-7 cells. (A) The experimental scheme for the treatment with 1 nM MBP for booster (+) or 2 nM MBP for up to 48 hours. The treatment of 1 nM MBP for booster (+) followed the same method as for booster (+) in Fig. 2B. In contrast, the experimental scheme for 2 nM MBP treatment involved no culture medium change and additional exposure. (B left panel) Western blotting of ER α and ER β . MCF-7 cells were treated with 1 nM MBP for booster (+) or 2 nM MBP. After chemical treatment for a total of 48 hours, total cell lysates were prepared, and Western blotting of antibodies specific for ER α , ER β , and β -actin was performed; β -actin was used as an internal loading control. Cell lysates prepared with human ER α or ER β cDNA-transfected cells were used as the positive control (indicated as Std.). (B, right panel) The band intensity of ER α (left panel) and ER β (right panel) was quantified by using ImageJ 1.46r software, and the values were normalized to β -actin. The data are expressed as the mean $\pm$ S.D. ($n = 3$) of the fold change from booster (+) cells (indicated as 1). (C) MCF-7 cells were exposed to 2 nM MBP in the presence or absence of 0.5 μ M PHTPP. After incubation for 48 hours, the cell proliferation was measured. The control sample was treated with vehicle alone (indicated as Ctl.). The data are presented as the mean $\pm$ S.D. ($n = 6$) as a percentage of the control. Significant differences [Student's t test for (B, right panel); one-way ANOVA, repeated (related) measures with Tukey-Kramer's post hoc test for (C)] compared with booster (+) (B) or control (C) are marked with asterisks (* $P < 0.05$). N.S., not significant.

whether the introduction of PHTPP abrogated the MBP-enhanced cell proliferation and found that MCF-7 cell proliferation stimulated by MBP was dose-dependently dampened by PHTPP (Fig. 8B). The effects of MCF-7 cells exposed to 1 nM MBP for a total of 48 hours with repeated exposure [i.e., booster (+): 1 nM MBP + 1 nM MBP] or without [i.e., booster (-): 1 nM MBP + vehicle instead of MBP] are shown in Figs. 4, 6B, 7, and 8B; however, at 24 hours after the first exposure to

Fig. 10. Summary of the effects of repeated exposure to MBP, an endocrine-disrupting chemical, on human breast cancer cells. ER α signaling tends to be abrogated by coexisting ER β in human breast cancers with no exposure to MBP. In this study, it was revealed that repeated exposure to MBP stimulated ERE-driven transcriptional activity and cell proliferation, even though repeated exposure to MBP induced the down-regulation of ER α . Mechanistically, the MBP-mediated stimulation of transcriptional activity/cell proliferation occurred in an ER β -dependent manner. These effects were selectively observed with MBP, but not with the parent compound, BPA.

MBP, the culture medium, including MBP, was replaced with fresh medium together with MBP or vehicle. Therefore, in this experimental regimen (Fig. 2B), one possible criticism might be that the apparent effects of repeated exposure to MBP, booster (+) versus booster (–), can be simply explained by the difference in the “total” exposure of cells to MBP. Thus, we analyzed the effects of 2 nM MBP on cells treated for 48 hours without the booster by a comparison of the effects of cells repeatedly exposed to 1 nM MBP [booster (+)] (see experimental regimen described in Fig. 9A). The obtained results of Western blotting indicated that in comparison with the booster (+) results with 1 nM MBP, the expression of both ER α and ER β proteins was significantly upregulated by 2 nM MBP treatment (Fig. 9B). Furthermore, it was unexpectedly revealed that an inactive effect of PHTPP (0.5 μ M) on the cell viability after treatment at 2 nM MBP for 48 hours without its booster was observed (Fig. 9C), implicating the involvement of the MBP-mediated ER α activation.

Discussion

An accumulation of evidence suggests that BPA has a carcinogenic activity in the human breast because of its tumor-promoting properties (Seachrist et al., 2016). We are continually exposed to environmental BPA. In the current study, we investigated the effects of repeated exposure of BPA, together with MBP, at concentrations similar to environmental levels on the human breast cancer MCF-7 cell line, and demonstrated that 1) “repeated” exposure to MBP, but not parent BPA, stimulated the proliferation of MCF-7 cells through the mitogenic activity of cell cycle acceleration and 2) the bisphenol BPA used the ER β subtype to induce mitogenic action.

It has been reported that BPA can be detected at concentrations between 0.3 and 40 nM in human biologic samples from fetal serum and maternal plasma (Schönfelder et al., 2002; Welshon et al., 2006; Vandenberg et al., 2010). The combination of the data in Figs. 3 and 5, indicated the following: 1) although BPA can act as a positive stimulator of MCF-7 cell proliferation, as well as ER/ERE-mediated transcriptional activity, the former effect by this bisphenol

at concentrations below 40 nM (Fig. 3A) may not be fully indicated by the data in Fig. 5, A and B, because reasonably high concentrations of BPA are required to induce the full activation of ER/ERE-mediated transcriptional activity (>500 nM); and, importantly, 2) it was revealed that in good accordance with the MBP-mediated stimulation of MCF-7 cell proliferation, which was observed at concentrations below 40 nM (i.e., physiologic concentrations), the MBP-mediated activation of ER/ERE-mediated transcriptional activity in MCF-7 cells was also seen at concentrations below 40 nM, which implied the occurrence of a key interaction between the MBP activation of ERs and MCF-7 cell proliferation. Although the detailed regulation machinery of the ER α / β protein associated with MBP exposure in MCF-7 cells is unknown at present, after consideration of these observations, it was suggested that MBP can use ER β (for which expression was unaffected) to induce its biologic effects (e.g., the stimulation of breast cancer cell proliferation) in ER α / β -positive cells after repeated exposure. Through the construction of the three-dimensional analysis models of MBP with human ER α / β , it was suggested that MBP, but not BPA, interacted with amino acid residues in ER α / β that were important for the binding of E2 in ERs (Baker and Chandsawangbhuwana, 2012). We have reported that the EC₅₀ values for ER α and ER β activation by MBP were 0.68 and 0.46 nM, respectively, in NIH/3T3 cells transfected with expression plasmids encoding human ER α or ER β (Yoshihara et al., 2004). Although we were unable to obtain the EC₅₀ values for the individual subtypes of ER α and ER β after the activation by MBP in MCF-7 cells, it was suggested that MBP may behave as an equipotent activator of human ER α / β .

In this study, it was also revealed that BPA itself can stimulate MCF-7 cell proliferation at concentrations below 40 nM; as recently reported, this effect of BPA might be mediated by nuclear ER-independent signaling pathways: 1) Shimohigashi and Matsushima's research group reported that BPA had a high affinity (K_d = 5.50 nM) for orphan nuclear receptor estrogen-related receptor γ and that the interaction between BPA and estrogen-related receptor γ may explain the biologic effects of low-dose BPA (Takayanagi et al., 2006; Okada et al., 2008); and 2) BPA targets G-protein coupled estrogen receptor (GPR30/GPER) expressed in the cell membrane to cause nongenomic estrogenic signaling (Dong et al., 2011; Ge et al., 2014). Exposure to environmental chemicals may be associated with many undesirable health outcomes; however, the negative effects of these environmental chemicals are generally weakened by drug-metabolizing enzymes (phase I and II reactions) after exposure. In the case of BPA, it has been reported that under in vivo conditions, BPA is readily converted to BPA-glucuronide (via phase II reaction); however, BPA-glucuronide has itself also been proven to be a possible active metabolite that induces adipogenesis in human and murine preadipocytes (Boucher et al., 2015). Although it has been suggested that ER β has a possible protective role against breast tumor progression, the accumulated evidence suggests that ER β also acts as a mediator of estrogen action, coupled with tumorigenesis, in breast cancer cells, and that its expression is remarkably upregulated in tamoxifen-resistant breast cancers (Dotzlaw et al., 1999; Speirs et al., 1999a,b; Power and Thompson, 2003; Hamilton et al., 2015; Ma et al., 2017). Collectively, including our findings here, it is suggested that the negative biologic effects of BPA are exerted through several

methods, such as through BPA itself and BPA metabolites, including BPA-glucuronide and MBP, and that the MBP-mediated estrogenic effects on breast cancer cells, which were possibly amplified by repeated exposure, were mediated by ER β (Fig. 10).

Authorship Contributions

Participated in research design: Takeda.

Conducted experiments: Hirao-Suzuki, Okuda.

Performed data analysis: Hirao-Suzuki, Takeda.

Wrote or contributed to the writing of the manuscript: Hirao-Suzuki, Takeda, Okuda, Takiguchi, Yoshihara.

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