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 (ERβ)–Dependent Manner

Masayo Hirao-Suzuki, Shuso Takeda, Katsuhiro Okuda, Masufumi Takiguchi, and Shin’ichi Yoshihara

Laboratory of Xenobiotic Metabolism and Environmental Toxicology, Faculty of Pharmaceutical Sciences, Hiroshima International University (HIU), Kure, Hiroshima, Japan (M.H.-S., S.T., M.T., S.Y.); and Department of Legal Medicine, Asahikawa Medical University, Asahikawa, Hokkaido, Japan (K.O.)

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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 ERα/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 ERα/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 ERα/β and to determine the particular ER subtype involved. We demonstrated that repeated exposure to MBP, but not to BPA, significantly downregulated ERα protein expression and stimulated the proliferation of MCF-7 cells through the activation of ERβ–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; Welchons 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 17β-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 coinubcation of BPA and liver S9 fraction, 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...
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 ERα and ERβ 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 ERα/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 ERα/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/ERα signal transduction pathways (Foster et al., 2001). Unlike ERα, the physiologic role of ERβ is not fully understood; however, the β-type ER is recognized as a functional repressor of ERα owing to its ability to heterodimerize with ERα 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 ERβ may be a tumor suppressor owing to its reduced expression during cancer development (Iwao et al., 2000). However, the accumulated evidence suggests that ERβ 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 ERα/β, and investigated the ER subtype that was involved in these effects. Our results indicated that repeated exposure to MBP, but not BPA, downregulated ERα protein expression and stimulated the proliferation of MCF-7 cells through the activation of ERβ.

Materials and Methods

Reagents. ICI 182,780 (fulvestrant) (purity, ≥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 (PHPTT) (purity, ≥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)bisphenol] (purity, ≥99%) (CAS number 80-05-7) was purchased from Nacalai Tesque (Kyoto, Japan). The chemical synthesis of MBP (purity, ≥98%) (CAS number 13464-24-9) was performed in accordance with previously reported methods (Yoshihara et al., 2004; Okuda et al., 2010). E2 (purity, ≥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 (MEMα) (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10 nM HEPES, 5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/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 MEMα (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10 nM HEPES, 5% dextran-coated charcoal-treated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). ICI 182,780, PHPTT, 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 PHPTP 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), for 24 hours of the first treatment, the culture medium was changed to fresh phenol red–free MEMα, 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 PHPTP. 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 × 104 cells/well) were seeded in 24-well plates containing MEMα. 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)-luciferase plasmid and 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 fresh 100 μl of passive lysis buffer (Promega, Madison, WI), from which 20 μl 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 ~5 × 103 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 × 105 cells/sample) and fixed in 70% ethanol at 4°C overnight. The fixed cells were harvested and resuspended in 450 μl of PBS containing 50 μg/ml propidium iodide (PI) (Nacalai Tesque) and 100 μg/ml RNase A (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 × 105 cells was counted per sample. The obtained results were analyzed by using ModFit LT version 3.0 (Verity Software House, Topsham, ME).
Antibodies and Western Blotting. Antibodies specific for ERα (ab79413; Abcam, Cambridge, MA), ERβ (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 ERα or ERβ cDNA were used as the positive control (indicated as Std.; Figs. 6a and 9B). SDS-PAGE/Western immunoblotting was performed based on previously described procedures (Suzuki et al., 2017b). The antibodies for ERα (1:2000 dilution), ERβ (1:2000 dilution), and β-actin (1:4000 dilution) were used as the primary antibodies for the detection of ERα, ERβ, 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 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: ERα (sense), 5'-GAA AGG TGG GAT ACG AAA AGA CC-3'; ERα (antisense), 3’-GCT GGA ATT ACC GCG GCT-5'; 18S-ribosomal RNA (sense), 5’-CGG CTA CCA CAT CCA AGG A-3'; and 18S-ribosomal RNA (antisense), 3’-GCT GGA ATT ACC GCG GCT-5'. The mRNA expression of ERα 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 elicted 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

ERβ as a Negative Regulator of ERα-Mediated Transcriptional Activity. It has been reported that estrogen signaling can be modulated by several methods; for
example, when ERα and ERβ are simultaneously expressed in cells, E2/ERα-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 ERα/ERβ 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 vitro conditions, that the expression of the individual ERs is one of the key determinants for the output of E2 signaling. ERα-positive MCF-7 cells produce endogenous E2, but at very low levels, to stimulate their own growth via E2/ERα and express the ERβ 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 ERα and ERβ, respectively, in the absence of exogenous E2. As shown in 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 μM, whereas PHTPP stimulated the transcriptional activity in a concentration-dependent manner up to 10 μM. These data strongly suggested that E2/ER-mediated estrogen signaling was triggered mainly by the ERα subtype and that ERβ exerted a negative impact on ERα in the conditions in which cells coexpressed both subtypes (Fig. 1A).

**MBP Stimulation of Breast Cancer Cell Growth through ERβ**

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 ERα/ERβ expression, which may be coupled with the dysregulation of estrogen receptor signaling. Therefore, we first investigated...
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 μM. 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 μM (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 ERα mRNA/Protein in a Repeated Exposure-Dependent Manner. It has been reported that the ERα 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.,...
Given that MBP behaves as an ERα ligand to induce similar biologic effects in E2, the protein expression of ERα may be downregulated after repeated exposure to MBP. Thus, we analyzed the protein expression of ERα, together with ERβ, 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 ERα/β expression were found relative to the control in either condition (booster +/−); however, the BPA metabolite MBP did reduce the expression of ERα in a treatment-dependent manner without significant alteration of the protein expression of ERβ, although there was a tendency for ERβ to be upregulated (Fig. 6, A and B).

Involvement of ERβ 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 (ERα and ERβ); for this, the MDA-MB-231 cell line, which is ERα

1999). Given that MBP behaves as an ERα ligand to induce similar biologic effects in E2, the protein expression of ERα may be downregulated after repeated exposure to MBP. Thus, we analyzed the protein expression of ERα, together with ERβ, 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 ERα/β expression were found relative to the control in either condition (booster +/−); however, the BPA metabolite MBP did reduce the expression of ERα in a treatment-dependent manner without significant alteration of the protein expression of ERβ, although there was a tendency for ERβ to be upregulated (Fig. 6, A and B). To determine whether MBP also affected ERα mRNA expression after repeated exposure, we performed real-time RT-PCR analysis of samples derived from both conditions (booster +/−) (Fig. 7). 1 nM MBP, but not 1 nM BPA, downregulated the mRNA expression of ERα in a repeated exposure-dependent way. Collectively, these data suggested that the protein expression ERα can also be downmodulated by MBP via a reduction in ERα mRNA.
negative and has a very low expression of ERβ, 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 ERβ was indeed involved in the MBP-induced transcriptional activation of ERβ/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 (0.1, 0.25, 0.5, and 1 μM), a selective antagonist of the ERβ 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 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).
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 ERα and ERβ proteins was significantly upregulated by 2 nM MBP treatment (Fig. 9B). Furthermore, it was unexpectedly revealed that an inactive effect of PHTPP (0.5 μM) 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 ERα 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 ERβ 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 ERα/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 ERβ (for which expression was unaffected) to induce its biologic effects (e.g., the stimulation of breast cancer cell proliferation) in ERα/β-positive cells after repeated exposure. Through the construction of the three-dimensional analysis models of MBP with human ERα/β, it was suggested that MBP, but not BPA, interacted with amino acid residues in ERα/β that were important for the binding of E2 in ERs (Baker and Chandsawangbhuvana, 2012). We have reported that the EC50 values for ERα and ERβ activation by MBP were 0.68 and 0.46 nM, respectively, in NIH/3T3 cells transfected with expression plasmids encoding human ERα or ERβ (Yoshihara et al., 2004). Although we were unable to obtain the EC50 values for the individual subtypes of ERα and ERβ after the activation by MBP in MCF-7 cells, it was suggested that MBP may behave as an equipotent activator of human ERα/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 (Kd = 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 ERβ has a possible protective role against breast tumor progression, the accumulated evidence suggests that ERα 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 ERβ (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, Yoshiharu.

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