Bisphenol AF as an Inducer of Estrogen Receptor β (ERβ): Evidence for Anti-estrogenic Effects at Higher Concentrations in Human Breast Cancer Cells

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Bisphenols are endocrine disruptors that are widely found in the environment. Accumulating experimental evidence suggests an adverse interaction between bisphenols and estrogen signaling. Most studies have performed experiments that focused on estrogen receptor (ER) engagement by bisphenols. Therefore, the effects of bisphenols on the expression of ERα (ESR1) and ERβ (ESR2) remain largely unknown. In the present study, we examined the effects of four bisphenols: bisphenol A (BPA), bisphenol B (BPB), bisphenol S (BPS), and bisphenol AF (BPAF), on estrogen signaling in two human breast cancer cell lines (MCF-7 and SK-BR-3). Among these bisphenols, BPAF up-regulated the expression of ERβ, and this was coupled with the abrogation of estrogen response element (ERE)-mediated transcriptional activities as well as the down-regulation of Cdc2 expression in MCF-7 cells, without influencing the expression of ERα. BPAF functioned as an agonist of ERα at lower concentrations (nanomolar order), but did not exhibit any modulatory action on ERα transiently expressed in SK-BR-3 cells in the presence or absence of 17β-estradiol (E2) at higher concentrations (micromolar order). The introduction of ERβ cDNA resulted in greater reductions in MCF-7 cell viability than with BPAF alone. Since ERβ is a suppressive molecule of ERα function, these results provide rational evidence for BPAF functioning as an anti-estrogenic compound via the induction of ERβ at higher concentrations.

Key words bisphenol AF; estrogen receptor β; anti-estrogenic compound; MCF-7 cell

Bisphenol A (BPA) (CAS No. 80-05-7) was invented in 1891, has since been used to create polycarbonate plastics and epoxy resins, and is a well-known endocrine-disrupting chemical (EDC).¹¹ Now the use of BPA is being restricted worldwide including Japan. Based on this circumstance, other bisphenols (i.e., bisphenol B (BPB) (CAS No. 77-40-7), bisphenol S (BPS) (CAS No. 80-09-1), and bisphenol AF (BPAF) (CAS No. 1478-61-1)) having structures/functions quite resemblable to BPA have been synthetized and utilized so far. However, at present the use of them is “not” prohibited. Thus, their possible adverse effects to humans are anticipated; indeed, they were detected in human biological samples.² Bisphenols (BPA, BPB, BPS, and BPAF) have been shown to exert positive effects on estrogen receptor (ER)α-mediated transcriptional activation in in vitro studies (i.e., nanomolar concentrations)³–⁷; however, it currently remains unclear whether these bisphenols affect the expression of ERα/β, and if this is coupled with the “modulation” of estrogen signaling. Therefore, more detailed toxicological data need to be acquired in order to establish the toxicological basis of bisphenols in vitro.

Although ER (ERα, ESR1) was considered to be the only receptor capable of mediating the effects of 17β-estradiol (E2) on target tissues based on its discovery in 1986,³⁹ ERβ (ESR2), a second type of ER, has since been identified in humans and other organisms.¹⁰–¹² The transcriptional effects of E2 are mediated by the ERs, ERα and ERβ, and the physiological roles of these ERs have been investigated. When ERα and ERβ are expressed together in the same cells, ERβ functions as a “repressor” of E2/ERα transcriptional activity through the formation of less active ERα/ERβ heterodimers.¹³–¹⁵ Thus, chemicals that have the potential to modulate the ratio of ERα/ERβ expression may be E2 action disruptors.

In the present study, we examined the effects of four bisphenols (BPA, BPB, BPS, and BPAF) on estrogen signaling in human breast cancer cells. Among these bisphenols, BPAF inhibited E2-activated transcriptional activity in ERα-positive MCF-7 cells (IC₅₀=19.3 µm), and this was coupled with the down-regulation of Cdc2, an ERα-regulated gene involved in cell cycle progression,⁶,¹⁷ without affecting ERα expression. The following results were obtained: i) ERβ expression (mRNA/protein levels) as well as its-regulated early growth response-1 (Egr-1) (mRNA), a transcription factor negatively involved for breast cancer cell proliferation, was up-regulated by BPAF only at micromolar concentrations in MCF-7 cells, ii) the transcription of human ERα, which is overexpressed in SK-BR-3 cells, was not modulated by BPAF in the presence or absence of E2, and iii) the ectopic expression of ERβ cDNA positively enhanced BPAF-mediated reductions in MCF-7 cell

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viability. Collectively, these results indicate that BPAF functions as an anti-estrogen for E2/ERα signaling through the up-regulation of ERβ at a higher concentration range in ERα-positive breast cancer cells.

MATERIALS AND METHODS

**Test Compounds** Experiments were performed using four bisphenols. BPA (2,2-bis(4-hydroxy phenyl)propane) (purity: >99%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BPAF (1,1,1,3,3,3-hexafluoro-2,2-bis(4-hydroxy phenyl)propane) (purity: >98%), BPB (2,2-bis(4-hydroxyphenyl)butane) (purity: >98%), and BPS (bis(4-hydroxyphenyl)sulfone) (purity: >98%) were purchased from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of the highest grade commercially available.

**Cell Culture** Cell culture conditions and methods were performed as described previously. Briefly, the human breast cancer cell lines, MCF-7 and SK-BR-3 (obtained from the American Type Culture Collection, Rockville, MD, U.S.A.) were routinely grown in phenol red-containing minimum essential medium (MEM)(Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 5% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin in a humidified incubator within an atmosphere of 5% CO₂ at 37°C. Prior to the chemical treatments, medium was changed to phenol red-free MEM α supplemented with 5% dextran-coated charcoal-treated serum, followed by the respective chemical treatments being tested. In experiments using breast cancer cells, cells were treated with bisphenols in the presence or absence of 1 nM E2. After 24 h, cell extracts were prepared using 100 µL of passive lysis buffer (Promega, Madison, WI, U.S.A.), and 20 µL was then applied to the firefly luciferase and Renilla luciferase assays (Dual-Luciferase Reporter Assay System, Promega) using the GloMax®-Multi Detection System (Promega). The ratio of firefly luciferase activity (expressed from reporter plasmids) to Renilla luciferase activity (expressed from pRL-CMV) in each sample served as a measure of normalized luciferase activity.

**Rеal-Time RT-PCR Analysis** Total RNA was prepared from MCF-7 cells using the RNeasy kit (Qiagen, Inc., Hilden, Germany) and purified using RNeasy/QLAamp columns (Qiagen, Inc.). In the real-time RT-PCR analysis of ERα, Cdc2, ERβ, Egr-1, and β-actin, cDNA was prepared via RT of total RNA using the ReverTra Ace® qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan). Real-time quantitative RT-PCR assays were performed with FastStart Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, U.S.A.) and the LightCycler® Nano (Roche Diagnostics, Mannheim, Germany). The primers for PCR on human β-actin, human Cdc2, human ERα, and human ERβ were taken from previous studies. The reaction conditions for all mRNAs were 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 58°C for 10 s, and 72°C for 15 s. ERα, ERβ, Cdc2, and Egr-1 mRNA levels were normalized to the corresponding β-actin mRNA levels.

**Transfection and Dual-Luciferase Reporter Assay** The day before transfection, human breast cancer cells (MCF-7 and SK-BR-3) were seeded (5×10⁴ cells/well) on 24-well plates containing MEMα. The transfection of each expression plasmid was performed using Lipofectamine® LTX with PLUS™ reagent (Invitrogen) according to the manufacturer’s instructions. DNA mixtures containing 300 ng of the (estrogen-responsive element, ERE)-luciferase reporter plasmid (pRL-CMV) in the plates. In experiments using SK-BR-3 cells, an expression plasmid carrying human ERα cDNA (100 ng) was also transfected (Fig. 3). Cells were washed with phosphate-buffered saline 24 h post-transfection and changed to phenol red-free MEMα supplemented with 5% dextran-coated charcoal-treated serum, followed by the respective chemical treatments being tested. In experiments using breast cancer cells, cells were treated with bisphenols in the presence or absence of 1 nM E2. After 24 h, cell extracts were prepared using 100 µL of passive lysis buffer (Promega, Madison, WI, U.S.A.), and 20 µL was then applied to the firefly luciferase and Renilla luciferase assays (Dual-Luciferase Reporter Assay System, Promega) using the GloMax®-Multi Detection System (Promega). The ratio of firefly luciferase activity (expressed from reporter plasmids) to Renilla luciferase activity (expressed from pRL-CMV) in each sample served as a measure of normalized luciferase activity.

**Antibodies and Western Immunoblot Analysis** Antibodies specific for ERβ (ab3576; Abcam, Cambridge, MA, U.S.A.) and actin (sc-1616 HRP; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used. Whole cell extracts were prepared using CelLytic™ MT Cell Lysis Reagent (Sigma-Al-

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**Fig. 1. Chemical Structures of 4 Bisphenols (BPA, BPB, BPS, and BPAF) Used in This Study**
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western immunoblotting was performed based on previously described procedure. Membranes were photographed using the Chemi Doc XRS plus system (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts of protein for each sample were confirmed by probing with actin. The quantification of band intensities was performed using ImageJ 1.46r software (http://imagej.nih.gov/ij/).

Cell Viability Analysis (MTS Assay) In the MTS assay, cells were seeded on 96-well plates at a density of 5×10³ cells/well, and the four bisphenols were added. After a 24h incubation, cell viability was analyzed using the CellTiter 96®
**Mechanisms of the BPAF Abrogation of Estrogen Signaling in Breast Cancer Cells**

There are at least two possibilities for the abrogation of ERα-mediated estrogen signaling; (1) ERα antagonism and (2) ERβ induction. We initially focused on possibility (1). In an attempt to clarify the effects of BPAF on ERα function, we used the human breast cancer cell line, SK-BR-3. In general, SK-BR-3 cells are recognized to be ER-negative. However, it has been pointed out that ERs are detected in the cells, implicating the importance of confirmation of ERs expression status. Thus, we first analyzed the expression status of ERα/ERβ expressed in SK-BR-3 cells used in this study. The data obtained indicated that no detectable signals of ERα are obtained, and that when ERβ is expressed in MDA-MB-231 cells (a known ERα−/ERβ− [very low] cell line) is set at 1.0, the expression levels of ERβ in SK-BR-3 cells were determined to be ca. 0.42. Thus, it is suggested that SK-BR-3 cells used in this experiment entirely lack or have very low levels of the expression of ERs. To simply understand the effects of BPAF on the ERα-mediated transcription, we thus utilized SK-BR-3 cells transfected with human ERα expression plasmid. As shown in Fig. 3, E2 (1 nM)-induced ERα activation (Control: 3.78-fold activation) was modestly suppressed by BPAF at higher concentrations. However, a clear concentration-dependent inhibitory effect by BPAF was not detected. Thus, at high concentrations (micromolar concentration range), BPAF may only exert weak interactive effects on ERα. We then focused on possibility (2). Among the 4 bisphenol analogs investigated at a concentration of 25 µm, only BPAF potently induced the ERβ transcript, and this was coupled with the induction of the Egr-1 transcript, a downstream gene of ERα (Figs. 4A, B). However, high concentrations (>150 µM) of BPAF appeared to be necessary to induce ERβ/Egr-1 transcripts (data not shown). The up-regulation of ERβ at the protein level was significantly greater by BPAF (25 µM) than by the vehicle control (Fig. 4C, left and right panels) compared to the vehicle treatment (Fig. 4C, left panel). These results suggest that among the four bisphenols investigated, BPAF specifically abrogated estrogen signaling and functioned as an anti-estrogen in ERα-positive MCF-7 cells at high concentrations.
Effects of Bisphenols on the Viability of MCF-7 Cells

MCF-7 cell growth depends on E2/ERα signaling, and ERβ, a suppressor of ERα, which is consequently maintained at low levels in cells. As shown in Fig. 4, BPAF functioned as an inducer of ERβ. Based on these lines of evidence, we hypothesized that anti-estrogenic compounds for ERα function may negatively modulate cell proliferation. Therefore, we assessed the IC₅₀ values of cell viability after individual treatments with the four bisphenols for 24 h. Among the bisphenols tested, BPAF decreased cell viability in a concentration-dependent manner with an IC₅₀ value of 28.1 μM, whereas the other bisphenols did not exert any inhibitory effects, even at a concentration of 50 μM (IC₅₀ values > 50 μM) (Fig. 5A). In accordance with cell viability studies, cell morphology was also typically affected by BPAF, which resulted in a rounder cell shape (Fig. 5B). Since the levels of ERβ induced are important for reducing cell viability, ERβ cDNA transfection may positively stimulate BPAF-mediated suppression in cell viability. As shown in Fig. 6, BPAF-mediated suppressive effects on cell viability were positively enhanced by ERβ cDNA transfection under conditions in which BPAF alone (no DNA control) as well as BPAF/mock transfection exerted similar effects on cell viability although ERβ cDNA-mediated additive effects were modest in degree (Fig. 6).

DISCUSSION

Although bisphenols including BPAF have been shown to function as activators of ERα under E2-depleted/low conditions, such as in HeLa cells (human cervical adenocarcinoma; negative for aromatase, an enzyme responsible for E2 production), this phenomenon was not observed in other cell lines including HepG2 (human hepatocellular carcinoma; positive for aromatase) and Ishikawa (human endometrial adenocarcinoma; positive for aromatase). We utilized MCF-7 cells, which produce E2 at pmol levels (i.e., 0.26 pM/10⁶ cells) and in the presence of exogenously added 1 nM E2, as expected, the stimulation of ERα by bisphenols was not detected in MCF-7 cells (Fig. 2). However, only BPAF down-modulated estrogen signaling (Fig. 2). We did not detect clear dose-dependent inhibitory effects of BPAF on the activation by 1 nM E2 of ERα, which is overexpressed in SK-BR-3 cells (Fig. 3). In addition, when performing this type of experiment in the absence of E2, there were no observable stimulating effects of BPAF on ERα at micromolar concentrations (data not shown).

Fig. 4. Effects of BPAF on the Expression of ERβ in MCF-7 Cells
(A, B) Real-time RT-PCR analyses of ERβ and Egr-1 transcripts 48 h after the treatment with vehicle (control) or 4 bisphenols (25 μM). (C, left panel) A Western immunoblot analysis of ERβ. MCF-7 cells were treated with 4 bisphenols (25 μM) or vehicle (control) for 48 h. Total cell lysates were prepared, and Western immunoblot analyses were performed using antibodies specific for ERβ and actin, respectively. Actin was used as an internal loading control. (C, right panel) The band intensity of ERβ (control lane as 1.0), which was quantified using ImageJ 1.46r software, is shown. Data are expressed as a fold induction from the vehicle-treated control (indicated as 1), as the mean ± S.E. (n=3). *Significantly different (p<0.05) from the vehicle-treated control (control). N.S., not significant.
viability, which was coupled with the down-regulation of the Cdc2 transcript, an ER\(\alpha\)-regulated gene involved in cell cycle control (Figs. 2, 4, 5). Furthermore, the ectopic expression of ER\(\beta\) resulted in greater reductions in cell viability than with BPAF alone transfected with the mock plasmid (IC\(_{50}\) values for 48 h: 19.0 vs. 16.7 \(\mu\)M) (Fig. 6). Thus, BPAF appears to function as an anti-estrogen, but not as an estrogen in the micromolar concentration range via the up-regulation of ER\(\beta\), an ER\(\alpha\) suppressive molecule.

In the present study, we demonstrated that BPAF was the most anti-estrogenic compound among the four bisphenols investigated in ER\(\alpha\)-positive MCF-7 cells. The mechanisms by which BPAF evokes these selective biological effects need to be elucidated. The potentially reactive trifluoromethyl group (–CF\(_3\)) in the BPAF structure may be a key factor; however, the other bisphenols lacked this halogenated moiety (Fig. 1). Lipophilicity is a physicochemical parameter and determinant of the toxicological fate of EDCs; e.g., the log \(P_{ow}\) of BPA (\(n\)-octanol/water) was found to be 3.32,\(^1\) while that of BPAF was 2.82.\(^{27}\) Thus, the toxicological impact of BPAF may be

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Fig. 5. Effects of Bisphenols on the Viability of MCF-7 Cells

(A) MCF-7 cells were exposed for 24 h to bisphenols (1, 2.5, 5, 10, 20, 25, 50 \(\mu\)M) to assess IC\(_{50}\) values for reductions in cell viability. After exposure, cell viability was measured according to the methods described in Materials and Methods. A control incubation was treated with vehicle alone. Data are expressed as a percentage of the vehicle-treated control as the mean±S.E. (n=6). (B) MCF-7 cells were treated with vehicle (control) and 25 \(\mu\)M bisphenols for 24 h prior the assessment of cellular morphology. Representative data images are shown. Images were taken at \(\times\)200 magnification.
expected to be lower than that of BPA. However, Waidyanatha et al.\(^{(28)}\) previously reported that BPAF was absorbed well after its administration to experimental animals, and its clearance from the hepatocytes of rats and mice was slower than that of BPA, implicating “abnormalities” in BPAF, which allows its unexpected toxicological effects. ER\(\beta\) is another type of ER that is more weakly expressed in the MCF-7 cell line than ER\(\alpha\). Accumulating experimental evidence demonstrated, for example, that in breast cancers, ER\(\beta\) inhibits MCF-7 cell proliferation (\textit{in vitro}) and tumor formation by MCF-7 cells in mouse xenografts (\textit{in vivo})\(^{(17,23)}\).

We previously reported that \(\Delta^9\)-THC had the potential to abrogate E2/ER\(\alpha\) signaling in MCF-7 cells by up-regulating ER\(\beta\), which resulted in the inhibition of cell proliferation, and also that \(\Delta^9\)-THC required the co-existence of ER\(\alpha\)/ER\(\beta\) to evoke its anti-proliferative effects\(^{(17,29)}\). Furthermore, Gustafsson and his colleagues showed that ER\(\beta\) suppressed E2/ER\(\alpha\)-mediated uterine progression in immature mice\(^{(30)}\). Although we observed the anti-estrogenic nature of BPAF in breast cancer cell lines (\textit{in vitro}), BPAF may induce unwanted events \textit{via} the perturbation of estrogen signaling after its accumulation in certain normal tissues. It has been reported that BPA and BPB can be metabolically converted to some metabolites by reactions in human S9 fractions.\(^{(31,32)}\) Given that the metabolites from BPA/BPB are also active, further studies are required to analyze the effects of these metabolites on ER\(\beta\) induction. Further studies are needed to demonstrate these possibilities.

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Conflict of Interest The authors declare no conflict of interest.

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