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Low-concentration BPF induced cell biological responses by the ERα and GPER1-mediated signaling pathways in MCF-7 breast cancer cells

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ABSTRACT

Bisphenol F (BPF), one of the alternatives to bisphenol A (BPA), can induce proliferation through the nuclear estrogen receptor ERα (estrogen receptor alpha) pathway in human breast cancer MCF-7 cells. However, the roles of membrane estrogen receptor GPER1 (G-protein-coupled receptor 1)-mediated signaling pathways in MCF-7 cell proliferation caused by BPF are unclear. The influence of BPF on MCF-7 cells was evaluated in terms of cell proliferation, intracellular calcium (Ca^{2+}) fluctuations, and reactive oxygen species (ROS) generation. The molecular mechanisms of the cellular responses to low doses of BPF were studied through detecting the activations of ERα and GPER1-regulated PI3K/PKB or AKT (phosphatidylinotidol 3-kinase/protein kinase B) and ERK1/2 (extracellular-signa1-regulated kinase 1/2) signals. At 0.01–1 μM, BPF significantly promoted cell proliferation and elevated the levels of intracellular ROS and Ca^{2+}. At these concentrations, BPF also significantly upregulated protein expressions of ERα, GPER1, c-myc, and cyclin D and phosphorylations of PKB and ERK1/2. Specific signal inhibitors decreased PKB and ERK1/2 phosphorylations and attenuated the effects of BPF. Silencing of GPER1 also significantly decreased BPF-induced cell proliferation. These results indicate that activating the GPER1-PI3K/PKB and ERK1/2 signals by low doses of BPF can regulate the response of MCF-7 cells and that ERα also influences the effects of exposure to BPF on the cells. The present study suggests a new mechanism by which BPF exerts relevant estrogenic action in cancer cells and also highlights the potential risks in using BPF as an alternative to BPA.

1. Introduction

BPF (bisphenol F), as one of the alternatives to bisphenol A (BPA), is widely used in the manufacture of polycarbonate plastics, epoxy resins, structural adhesives, water pipes, and food contact materials (Wang et al., 2014). The restrictive use of BPA has made BPF one of the major alternatives, resulting in its intensive production and application (Qiu et al., 2018). Given its extensive use, BPF was found in different environmental media, including dust, soil, water and sediment (Song et al., 2014; Yang et al., 2014; Lee et al., 2015).Its widespread use in food packaging and a range of household materials and products means that BPF is also present at varying concentrations in food and drinking water (Wang et al., 2014). An average BPF concentration of 0.18 μg/L was found in food samples and soft drinks (Huang et al., 2016). BPF has comparable frequency and even greater concentrations than those of BPA in the urine of adults not exposed to BPF occupationally (Ye et al., 2015).

Despite its large-scale use, however, toxicological information on BPF is limited. The available data suggest that BPF usually displays BPA-like toxicity in cell lines: the potency may be weaker, similar to, or

Abbreviations: ERα/β, (estrogen receptor alpha/beta); GPER1, (G-protein-coupled receptor 1); PI3K/PKB, (phosphatidylinotidol 3-kinase/Protein kinase B); ERK1/2, extracellular-signa1-regulated kinase; ICI, ICI182780; WM, wortmannin; MKH, MK-2206 2HCl; U, U0126; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5)-dimethythiahiazo(-z-y1)−3,5-di-phenytetrazoliumromide; t-BHP, tert-butyl hydroperoxide; MAPK, (mitogen-activated protein kinase); EGFR, epidermal growth factor receptor; ROS, reactive oxygen species; Ca^{2+}, calcium ion; BPF, bisphenol F; BPA, bisphenol A; E2, 17β-estradiol; DMSO, dimethyl sulfoxide; cdk-4/6, (cyclin-dependent kinase-4/6); PKG, Protein Kinase G; TDP, thiodiphenol

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higher than that of BPA, depending on the cell models and the toxicity endpoints (Molina-Molina et al., 2013; Svajger et al., 2016; Macczak et al., 2017; Russo et al., 2018; Qiu et al., 2018). Rochester and Bolden (2015) made a systematic review of hormonal activity of BPF. Strong evidence showed that BPF had estrogenic and androgenic activities, and their potencies are similar to those of BPA, supporting *in vivo* observations. Some studies have also found that BPF is anti-androgenic. Huang et al. (2016) found that BPF showed anti-androgenic activity by binding to the androgen receptor (AR). In a mouse FeTA model under basal conditions, BPF presented a similar dose-effect relationship to that shown by BPA in reducing the testosterone secretion amounts by mouse fetal testes (Eladak et al., 2015). In cultured human fetal testes, BPF also reduced basal testosterone secretion and showed anti-androgenic effects. Besides, BPF was also more sensitive than BPA in decreasing basal testosterone secretion at 10^{-6} M after 48 h of exposure (Desdoits-Lethimonier et al., 2017). Recent studies have shown that, similar to BPA, BPF has the potential to interfere with the thyroid hormone (TH) signaling pathway (Zhang et al., 2017, 2018; Zhu et al., 2018) and disrupt the normal endocrine system function (Eladak et al., 2015). BPF also showed other *in vitro* effects such as cytotoxicity and DNA damage (Rochester and Bolden, 2015). Compared with BPA, BPF is less toxic to HepG2 (Audebert et al., 2011). However, BPF had genotoxic effects that were not found with BPA (Audebert et al., 2011). Therefore, BPF may not be a safe alternative to BPA (Eladak et al., 2015)

GPER1 (G-protein-coupled receptor 1), also named GPR30, is a membrane estrogen receptor, which is structurally unrelated to nuclear estrogen receptor (ER) (Barton et al., 2018). GPER1-mediated mechanisms have recently been reported to be important in governing the action of estrogen and in different physiological functions (Sanchez et al., 2016; Smith et al., 2016; Lei et al., 2017a). In both cancerous and normal cells of mammary glands, BPA can stimulate such cellular effects as cell proliferation either via the traditional genomic signaling pathway by binding to the nuclear receptor ERα/β or via non-genomic responses by binding to GPER1 (Molina-Molina et al., 2013; Sanchez et al., 2016; Xu et al., 2017; Murata and Kang, 2018): BPF can also induce human breast cancer cells to proliferate by binding to nuclear ERα (Kim et al., 2017; Mesnage et al., 2017) but whether BPF can act also via non-genomic responses through GPER1-mediated signaling pathways remains uncertain.

Exposing MCF-7 cells to low doses of BPF for 24 h increased their viability, induced the ROS (reactive oxygen species) generation, and caused the levels of Ca^{2+} (calcium) to fluctuate (Lei et al., 2018). However, neither the relationships between these effects and the duration of exposure to BPF nor the signalling mechanisms through which the effects are induced have been studied in detail. However, whether BPF can activate the GPER1-mediated PI3K/PKB (also called AKT) (phosphatidylinotidol 3-kinase/protein kinase B) pathway and ERK1/2 (extracellular-signal regulated kinase) pathways, both of which influence cell growth induced by BPF, is unclear.

It was against this background that the present study sought to (1) evaluate the effects of different doses of BPF on cell proliferation, intracellular ROS generation and Ca^{2+} fluctuation following different durations of exposure; (2) investigate, by western blot analysis, the effects of low doses of BPF on the expression of ERα, c-myc, cyclin D, and GPER1 and on the phosphorylations of PKB and ERK1/2 in MCF-7 cells; and (3) determine whether low doses of BPF regulate cellular responses via those signaling pathways using specific inhibitors and RNA interference. The results will provide fundamental data for in-depth evaluation of potential risks to human health from BPF.

2. Materials and methods

2.1. Reagents

BPF (more than 99.0% pure, CAS No.620–92-8; EC No. 210–658-2) was purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan) and its stock solutions were dissolved in DMSO (dimethyl sulfoxide, 99.5%, AMRESCO, Radnor, Pennsylvania, USA) and kept at −20 °C. Fluo3-AM was purchased from Dojindo (Kumamoto, Japan); and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4,5)-dimethythiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT), estradiol (E2), and tert-butyl hydroperoxide (t-BHP) were purchased from Sigma (Saint Louis, Missouri, USA). GPER1, p-ERK1/2, ERK1/2, p-PKB and PKB antibodies were purchased from Cell Signaling (Beverly, MA, USA). C-myc and cyclinD antibodies were purchased from Millipore (Darmstadt, Germany); and GAPDH antibody, from Multisciences Biotechnology (Hangzhou, China). Inhibitors were bought from the following sources: ICI (ICI182780), Sigma (Saint Louis, Missouri, USA); G-15, Cayman (USA); WM (wortmannin), MKH (MK-2206 2HCl) and U (U0126), Selleck (USA). The other reagents used in this study are all analytically pure unless specified otherwise.

2.2. Maintenance and treatment of MCF-7 cells

The cell line used in the experiment is human breast cancer MCF-7 cells, obtained from the ATCC (American Tissue Culture Collection, Rockville, Maryland, USA). MCF-7 cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 supplemented with 10% FBS (fetal bovine serum), in details described by Lei et al. (2017b). The exponential growth phase cells were used for the experiments, which followed the same methods described in our earlier studies (Lei et al., 2018). In this study, solvent control was 0.1% DMSO, and the positive controls were 400 μM of t-BHP for the experiments on ROS and 10^{-8} M of E2 for the experiments on cell viability.

2.3. Cell viability assay

The assay for cell viability used the same MTT methods as those described previously (Lei et al., 2018). Cells seeded in 96-well plates and cultured for 24 h were treated with different concentrations of BPF (0.00001–100 μM) for 24 h, 48 h, or 72 h and then incubated with 100 μL of MTT (1 mg mL^{-1}) medium in the dark at 37 °C for 4 h. Cell viability (the number of viable cells as a percentage of viable cells in the control) was calculated based on optical densities of the treatment group and the solvent-control group.

2.4. Reactive oxygen species detection

The measurement of ROS involved an ROS assay probe, DCFH-DA, and the process was the same as that described earlier (Lei et al., 2018). Briefly, the treated cells were incubated with DCFH-DA for 30 min at 37 °C. The fluorescence was measured by fluorescence microscope, and the fluorescence intensities were analyzed using Image-Pro Plus ver. 6.

2.5. Detection of intracellular Ca^{2+}

Calcium content was detected by a fluorescent probe, Fluo-3/AM, as described elsewhere (Lei et al., 2018). Briefly, the treated cells were incubated with Fluo 3-Am and F-127 solutions for 40 min at 37 °C. Then, the fluorescence was measured using a fluorescence microscope, and the fluorescence intensities were analyzed using Image-Pro Plus ver. 6.

2.6. Western blot analysis

The MCF-7 cells were collected after 24 h of exposure. Western blot analysis was carried out the same way as described previously (Lei et al., 2017a). Briefly, both total proteins and nuclear protein samples were collected. The main primary antibodies included monoclonal antibodies ERα (1/1000), p-ERK1/2 (1/2000), p-PKB (1/2000), c-myc (1/1000), and cyclin D (1/1000) and a polyclonal antibody, GPER1 (1/250). For the experiments on inhibition, the cells were pretreated with