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Bisphenol AF exerts estrogenic activity in MCF-7 cells through activation of Erk and PI3K/Akt signals via GPER signaling pathway

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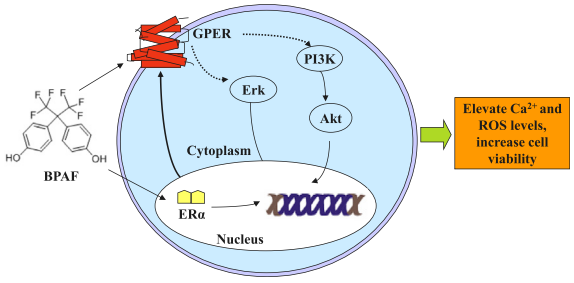
HIGHLIGHTS

- BPAF promotes cell proliferation, and elevates ROS and Ca^{2+} levels in MCF-7 cells.
- BPAF activates PI3K/Akt and Erk signaling pathways via GPER.
- Activation of GPER mediated signals stimulates BPAF induced cell biological effects.
- ERα plays a key role in cell biological effects induced by BPAF.
- BPAF can exert estrogenicity by interactions between ERα and GPER mediated signals.

GRAPHICAL ABSTRACT



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ABSTRACT

The negative health effects of bisphenol A (BPA) due to its estrogenic activity result in the increasing usage of alternative bisphenols (BPs) including bisphenol AF (BPAF). To comprehensive understand health effects of BPAF, the MCF-7 cells were used to investigate the effects of BPAF on cell proliferation, intracellular reactive oxygen species (ROS) formation, and calcium ion (Ca^{2+}) level. The molecular mechanisms of cell biological responses caused by BPAF were investigated by analyzing target protein expression. The results showed that low-concentration BPAF induces significant effects on MCF-7 cells, including promoting cell proliferation and elevating intracellular ROS and Ca^{2+} levels. BPAF in low concentration significantly enhances the protein expression of estrogen receptor α (ERα), G protein-coupled receptor (GPER), *c*-Myc, and Cyclin D1, as well as increases phosphorylation levels of protein

Abbreviations: BPA, bisphenol A; BPAF, bisphenol AF; CCK-8, cell counting kit-8; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; ER, estrogen receptor; Erk, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPER, G protein-coupled receptor; ICI, ICI182780; MK, MK-2206 2HCl; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; ROS, reactive oxygen species; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siGPER, siRNA targeting GPER; siNC, siRNA vector; siRNA, small interfering RNA; *t*-BHP, *tert*-butyl hydroperoxide; Wor, wortmannin.

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kinase B (Akt) and extracellular signal-regulated kinase (Erk) in MCF-7 cells. After the addition of ERα, GPER, and phosphatidylinositide 3-kinase (PI3K) inhibitors, phosphorylations of Erk and Akt were both inhibited. In addition, specific signal inhibitors significantly attenuated the effects of BPAF. Silencing of GPER also markedly decreased BPAF induced cell proliferation. The present results suggested that BPAF can activate PI3K/Akt and Erk signals via GPER, which, in turn, stimulate cellular biological effects induced by BPAF. ERα also plays a critical role in BPAF induced cellular biological effects.

1. Introduction

Bisphenol AF (BPAF), i.e., six fluorinated bisphenol A, is a bisphenol A (BPA) fluorinated derivative and has widespread application as a monomer in polycarbonate copolymers, electronic materials, and so on (Song et al., 2012). BPAF is now also recognized as one of the replacements for BPA in the production polycarbonate plastic and resins because the concerns regarding the toxicological effects of BPA have grown after the usage of the chemical in a number of consumer products during the past few decades (Liu et al., 2017). BPAF has become an emerging environmental pollutant (Ihde et al., 2018; Liu et al., 2017; Wang et al., 2017). For example, the highest levels of 15,300 ng/L in water, 2000 ng/g dry weight (dw) in sediment, and 739 ng/g dw in indoor dust were observed (Song et al., 2012). In recent years, BPAF has been found in different environmental media (Liao et al., 2012; Liu et al., 2017; Jin and Zhu, 2016; Song et al., 2012; Sun et al., 2018), foodstuffs (Cesen et al., 2016; Liao and Kannan, 2013; Mandrah et al., 2017) and biological samples (Wang et al., 2017) including blood of adults (Jin et al., 2018) with the mean concentration of 0.073 ng/mL, fetal cord blood (Ihde et al., 2018) with 57% detection rate, breast milk at concentrations ranging from ND to 0.052 μg/L (Niu et al., 2017), and urine samples at concentrations of ND-0.173 ng/mL (Yang et al., 2014). As an environmental estrogen, BPA has a similar potency to that of E2 (estradiol) in stimulating some cellular responses (Mesnage et al., 2017). However, compared with BPA, there is little information on the toxicity of the alternative BPAF, even at the *in vitro* level (Okazaki et al., 2018).

The chemical activity structure correlation analysis indicated that BPAF had stronger estrogen activity than BPA (Maruyama et al., 2013). Different methods, including *in vitro*, *in vivo*, and molecular docking, also showed that the estrogenic activity of BPAF is stronger than that of BPA (Li et al., 2012; Lei et al., 2017a; Mesnage et al., 2017; Mu et al., 2018). Similar to BPA, BPAF also can bind to the estrogen receptor (ER) ligand-binding domain and has agonistic activity for both ERα and ERβ with molecular dynamic simulations (Li et al., 2018). BPAF binds to ERα approximately 20 times more effectively than BPA and to ERβ almost 50 times more effectively as shown *in vitro* assays (Matsushima et al., 2010). In addition, BPAF can function as an agonist of ERα at nanomolar concentrations and as an antiestrogenic compound via the induction of ERβ1 at micromolar order in human breast cancer cells (Okazaki et al., 2017). BPAF can exhibit genomic effect by binding to or activating the nuclear ERα (Li et al., 2018). A recent study showed that BPAF also exerts nongenomic effects via the member G protein-coupled receptor (GPER) pathway and displays much stronger (9-fold) binding affinity than BPA (Cao et al., 2017). Except for estrogenic effects, BPAF also interacts with other receptors such as the androgen, gluocorticoid, thyroid, and human pregnane X receptors and exhibits different endocrine disturb effects (Feng et al., 2012, 2018; Fic et al., 2014; Kwon et al., 2016; Sui et al., 2012). In addition, BPAF shows more strongly oxidative damage in an *in vitro* cell model and higher lethality and developmental effects in a zebrafish embryo model compared with BPA (Maćczak et al., 2017; Mokra et al., 2018; Moreman et al., 2017; Russo et al., 2018). Moreover, BPAF has stronger bioaccumulation ability (Wang et al., 2017) and is more resistant to degradation than BPA (Choi and Lee, 2017). Therefore, BPAF was an endocrine disruptor more harmful than BPA.

It has been documented that BPAF could bind strongly to nuclear ERα or ERβ and function as an environmental estrogen. However, more recent evidence showed that estrogens can present a nongenomic response initiated at the cell membrane by binding to or activating the member GPER (Romano and Gorelick, 2018). The activation of GPER can rapidly activate downstream extracellular signal-regulated kinase (Erk) and phosphatidylinositide 3-kinase/protein kinase B (PI3K/Akt) signals that facilitate proliferation or other biological effects of breast cancer cells (Barton et al., 2018). A recent study also found that BPAF can promote cell migration and elevate intracellular calcium ion (Ca^{2+}) levels by member receptor GPER pathway in human breast cancer SKBR3 cells (Cao et al., 2017). However, whether BPAF can activate Erk and PI3K/Akt signals via GPER, which influences the cell biological response like cell proliferation, intracellular reactive oxygen species (ROS) levels induced by BPAF, is still not clear.

In the present study, human breast cancer MCF-7 cells were used as the experiment cell model, because both ERα and GPER have positive expressions in this cell line. The aims of the study are to (1) assess the effect of different concentrations of BPAF on cell proliferation, intracellular ROS, and Ca^{2+} concentration changes under different exposure times; (2) examine the ability of BPAF to activate PI3K/Akt and Erk signaling pathways mediated by ERα and GPER; and (3) analyze the roles of the main targets in low-concentration BPAF induced biological responses of breast cancer cells.

2. Materials and methods

2.1. Reagents and materials

BPAF (Cas No. 1478-61-1, more than 98% pure) was bought from Tokyo Chemical Industry (TCI, Tokyo, Japan). MCF-7 cells were obtained from American Tissue Culture Collection (Rockville, MD, USA). Fluo-3/AM and cell counting kit-8 (CCK-8) were bought from Dojindo (Kumamoto, Japan). Mammalian Protein Extraction Reagent (M-PER) and Nuclear and Cytoplasmic Extraction Reagent (NE-PER) were purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). *Tert*-butyl hydroperoxide (*t*-BHP) and 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Sigma (Saint Louis, MO, USA). Detailed description of antibodies, inhibitors, and other materials used in this study can be found in our previous studies (Lei et al., 2017b, 2018a).

2.2. CCK-8 cell proliferation assay

Routine cell maintenance was carried out according to the

methods in our previous study (Lei et al., 2017a). The CCK-8 method as described earlier (Lei et al., 2017a) was used to detect cell proliferation. Briefly, MCF-7 cells were treated with BPAF dissolved in dimethyl sulfoxide (DMSO) in the concentrations ranging from 0.01 nM to 100,000 nM. Negative control consisted of 0.1% DMSO and positive control contained 10^{-8} M E2. Thereafter, CCK-8 was added. The optical density was measured at a wavelength of 450 nm with use of a spectrophotometric plate reader to indirectly identify the number of viable cells.

2.3. ROS detection

The intracellular ROS production induced by BPAF was determined using the fluorescent probe and the specific detection process was described earlier (Lei et al., 2018b). Briefly, the cells were exposed to BPAF (0.01–100,000 nM) and 0.1% DMSO (negative control) for 1, 3, and 24 h *tert-butyl*-Hy6dropheroxide (*t*-BHP) with 200 μM as the positive control-treated cells only for 1 h. After exposure, MCF-7 cells were incubated with DCFH-DA. The fluorescence was detected using a fluorescence microscope, and the fluorescence intensity was analyzed.

2.4. Measurement of intracellular Ca^{2+} levels

The measurement of intracellular Ca^{2+} level was carried out using a fluorescent probe, namely Fluo-3/AM, and the specific measurement method was described in our previous study (Lei et al., 2018b). Briefly, the cells were exposed to BPAF (0.01–10000 nM) and 0.1% DMSO (negative control) for 1, 3, and 24 h, respectively. Then, the cells were incubated with 5 μM Fluo-3/AM and 0.05% (wt/vol) F-127 solutions in D-Hanks' balanced salt solution at 37 °C for 40 min, which was determined by the time course experiment for the Fluo-3/AM probe incubation (Fig. S1). After that, the cells were observed using a fluorescence microscope and fluorescence intensity was analyzed.

2.5. Western blot

MCF-7 cells were collected after exposure to BPAF (0.01–10000 nM) and 0.1% DMSO (negative control) for 24 h. The specific experiment was described in our previous study (Lei et al., 2017b). Briefly, the nuclear protein of ERα and total protein samples of other targets were obtained by NE-PER and M-PER, respectively. Equal amounts (20 μg) of protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred from the gels onto nitrocellulose membranes. The used specific monoclonal or polyclonal antibodies including *anti*-ERα (1:1000), *anti*-GPER (1:2000), *anti*-Erk, *anti-p*-ERK (1:1000), *anti*-Akt, *anti-p*-Akt (1:1000), and *anti-c*-Myc, *anti*-Cyclin D1, or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000).

To study the effect of inhibitors on target signals, the cells were pretreated for 1 h with different target inhibitors including GPER inhibitor G15, ERα inhibitor ICI 182,780 (ICI), PI3K inhibitor wortmannin (Wor), *p*-Akt inhibitor MK-2206 2HCl (MK), and *p*-Erk inhibitor U0126. Then the cells were exposed to BPAF (10 nM) and 0.1% DMSO (negative control) for 24 h. Thereafter, protein samples were collected and protein expression was analyzed similar to the methods by Lei et al. (2017b). The experiments were repeated three times.

The used inhibitor concentrations of ICI and G15 were 1 and 10 μM, while Wor, U0126, and MK were all 10 nM because at these concentrations, cell viability (data were not shown) were not markedly influenced by these inhibitors but corresponding protein expressions were inhibited by them (Fig. S2).

2.6. Small-interfering RNA-mediated silencing of GPER in MCF-7 cells

To further elucidate the role of GPER in the MCF-7 cell proliferation caused by low-concentration BPAF, the GPER small interfering RNA (siRNA) interference technique was used in this study. The siRNA targeting GPER (siGPER) and a nonspecific siRNA vector (siNC) were synthesized at Shanghai GenePharma Co. Ltd. The specific method of plasmid transfection is given by our previous study (Lei et al., 2018a).

To obtain a suitable transfection time, the cells were incubated with positive-control plasmid siGAPDH and non-specific siRNA vector (siNC) for 24, 48 and 72 h, respectively. The results found that the expression of GAPDH protein decreased when the cells were incubated with siGAPDH for 48 or 72 h (Fig. S3). Therefore, 48 h was set as incubation time in the subsequent interference plasmid siGPER transfection experiments.

MCF-7 cells were treated with siNC or siGPER and cultured for 48 h; after incubation, the cells were further treated with 10 nM BPAF for 24 h. Protein expression was analyzed by western blot.

2.7. Data analysis

The present data were expressed as means ± SD (standard deviation). The differences between BPAF groups and the negative control were tested using one-way analysis of variance with specific mean comparisons by Dunnett's test. A Student's *t*-test was used to analyze the difference between two groups. Statistically significance was set at $p < 0.05$.

3. Results and discussion

3.1. Cell proliferation

The E2 as positive control significantly increased MCF-7 cell proliferation (Fig. 1). Compared with DMSO, BPAF (ranging from 1 to 100 nM) treatment for 24, 48, and 72 h caused a significant increase in cell proliferation ($p < 0.01$), and BPAF at 25,000–100,000 nM significantly decreased the cell proliferation. When MCF-7 cells were exposed to 25,000 nM BPAF for 24, 48, and 72 h, the cell proliferation was decreased by 16%, 44%, and 53%, respectively. This indicated that the toxicity of BPAF on MCF-7 cells increased with exposure time. The calculated IC50 (50% inhibition concentration) values of BPAF for cell proliferation are 14813 nM for 24 h, 34579 nM for 48 h, and 32062 nM for 72 h according to the fitting equations between exposure concentrations and inhibition rate of BPAF on cell proliferation (Table S1). Our results observed that lower concentration exposure to BPAF can increase the cell proliferation and higher concentration exposure to BPAF can attenuate survival rates of MCF-7 cells. In addition, the exposure to BPAF for 24 h induced a significantly increase in lactate dehydrogenase (LDH) release when exposure concentrations are higher than 10,000 nM (Fig. S4). BPAF at these levels also significantly inhibit cell proliferation. LDH release can be detected as a cell membrane damage indicator induced by chemicals, indicating that membrane damage of MCF-7 cells induced by BPAF is related to cell survival.

BPA in low concentrations can promote estrogen-sensitive tumor cell proliferation by activating nuclear receptor ERα or membrane receptor GPER-mediated signaling pathways (Pupo et al., 2012). Similar to BPA, BPAF can promote proliferation of estrogen-sensitive tumor cells. A recent study found that BPAF can induce MCF-7 cell proliferation via the ERα pathway in a transcriptome experiment (Mesnage et al., 2017). However, whether BPAF can induce MCF-7 cell proliferation via the GPER pathway remains

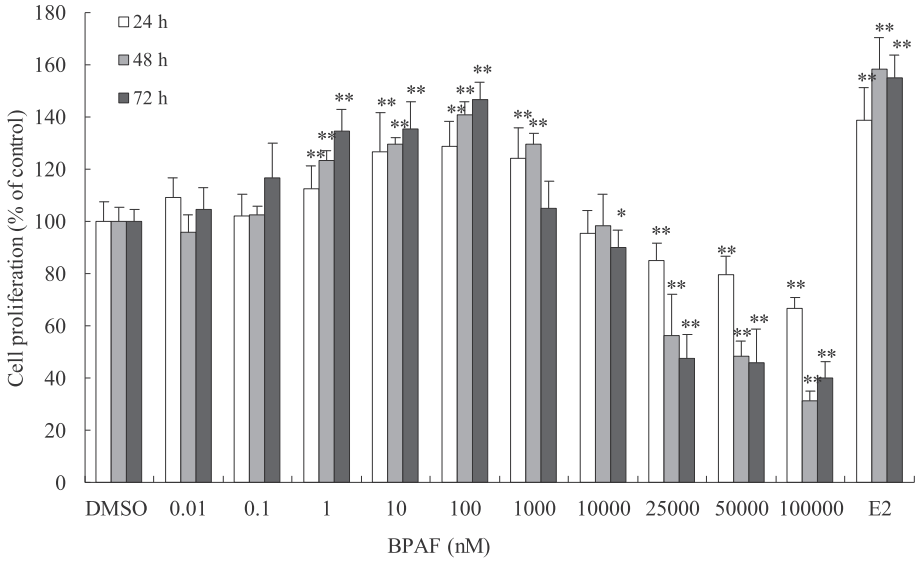


Fig. 1. Effects of BPAF (0.01–100000 nM) on the proliferation of MCF-7 cells. Data points represent mean ± SD of three independent experiments. Asterisks show statistically significant differences with respect to corresponding negative control (DMSO) (*$p < 0.05$, **$p < 0.01$).

unclear. Therefore, the role of GPER regulated signaling pathways in BPAF induced cell proliferation should be further studied.

3.2. ROS formation

DCFH-DA based on ROS detection was used to determine ROS levels induced by BPAF in MCF-7 cells. DCFH-DA can be deacetylated by intracellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) which can further be oxidized to the 2′,7′-dichlorofluorescein (DCF) with high fluorescence in the presence of ROS. Therefore, DCF fluorescence intensity can reflect intracellular ROS level. From Fig. 2, we can find that the positive control *t*-BHP markedly elevated intracellular ROS levels. In Fig. 2, BPAF exposure for 24 h induced a marked increase in ROS generation in all treatment groups compared with the control in MCF-7 cells. A concentration-dependent manner was observed at the exposure concentrations of 0.01–25,000 nM. Also, BPAF treatment for 1 and 3 h caused a concentration-dependent increase in intracellular ROS levels at the exposure concentrations of 10–100,000 nM. In addition, BPAF at the concentrations of 10–10,000 nM induced intracellular ROS increase in a time-dependent manner for 1, 3, and 24 h. The representative fluorescence microscopy images showing ROS formation in MCF-7 cells after treatment with BPAF (10 nM and 25000 nM) for 24 h are listed in Fig.S5.

In the present study, the results showed that BPAF in the whole range of the concentrations studied increased ROS level, whereas at low and high concentrations it increased and decreased cell proliferation, respectively. Many studies have shown that estrogens could cause ROS formation in estrogen-sensitive tumor cells and induce cells to proliferate (Deng et al., 2018; Mahalingaiah and Singh, 2014). Excessive ROS production may be one of the mechanisms by which higher-concentration BPAF induces cytotoxicity (Ding et al., 2017). It has been reported that BPA at higher

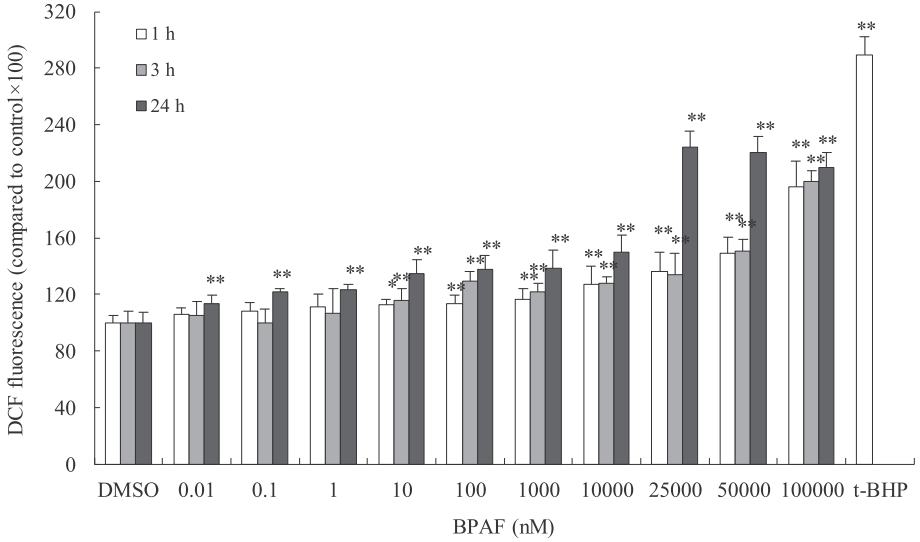


Fig. 2. ROS formation characterized by DCF fluorescence in MCF-7 cells after treatment with BPAF (0.01–100000 nM) for 1, 3, and 24 h. Columns represent the mean of three independent experiments ± SD. Asterisks show statistically significant differences with respect to corresponding negative control (DMSO) (*$p < 0.05$, **$p < 0.01$).

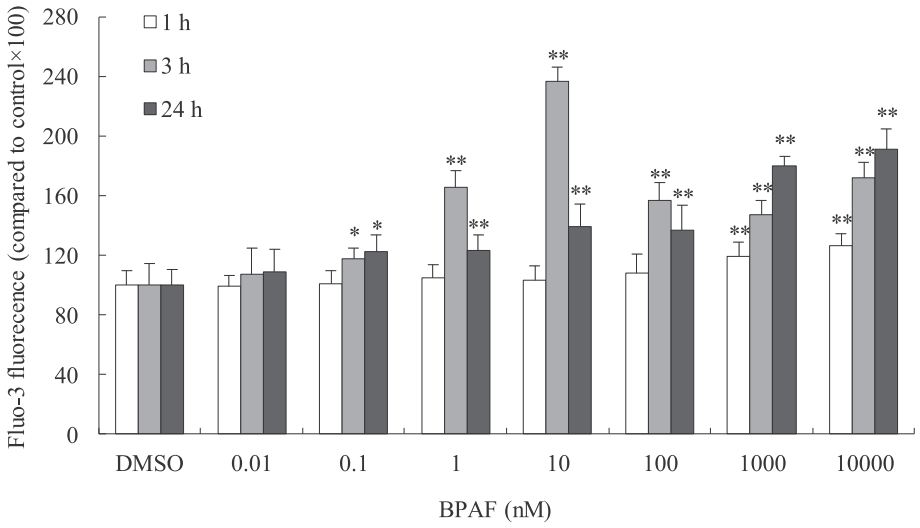


Fig. 3. Ca^{2+} fluctuation characterized by Fluo-3 fluorescence in MCF-7 cells after treatment with BPAF (0.01–10000 nM) for 1, 3, and 24 h. All values are expressed as mean ± SD of three independent experiments. Asterisks show statistically significant differences with respect to corresponding negative control (DMSO) (*$p < 0.05$, **$p < 0.01$).

concentrations (10^{-5} M) can cause oxidative stress and inhibit cell proliferation, while *N*-acetylcysteine (NAC), the ROS scavenger, effectively blocked these effects (Ge et al., 2014). In our previous study (Lei et al., 2018b), it also observed that ROS plays an important role in BPAF-mediated viability of MCF-7 cells. These results indicated the change of intracellular ROS levels induced by BPAF is closely related to MCF-7 cell survival.

3.3. Effects of BPAF on fluctuation of intracellular Ca^{2+} level

To measure the intracellular Ca^{2+} levels, Fluo-3/AM assay was carried out. Inside cells, Fluo-3/AM is hydrolyzed by cytosolic esterases to Fluo-3, which is effectively combined with Ca^{2+} to form Fluo-3-Ca^{2+}. Therefore, the fluorescence intensity of Fluo-3 can be used to evaluate intracellular Ca^{2+} level. The Fluo-3 fluorescence intensity data suggested that BPAF levels higher than 0.1 nM can significantly increase intracellular Ca^{2+} levels in a concentration-dependent manner exposure for 24 h (Fig. 3). When exposure time was 3 h, BPAF at lower concentrations significantly increases intracellular Ca^{2+} levels in a concentration-dependent manner up to 10 nM (by 2.5-fold), while the increase trend in Ca^{2+} levels is decreasing at the exposure concentration of BPAF greater than 10 nM. BPAF treatment for 1 h significantly increases Ca^{2+} levels only at the two highest exposure concentrations of 1000 and 10000 nM compared with negative control.

As a second messenger, Ca^{2+} plays key roles in regulating cellular processes such as gene expression, proliferation, and differentiation (Tajbakhsh et al., 2018). Many studies showed that Ca^{2+} regulates cellular growth via its interaction with many intracellular proteins and cellular signaling transduction pathways (Han et al.,

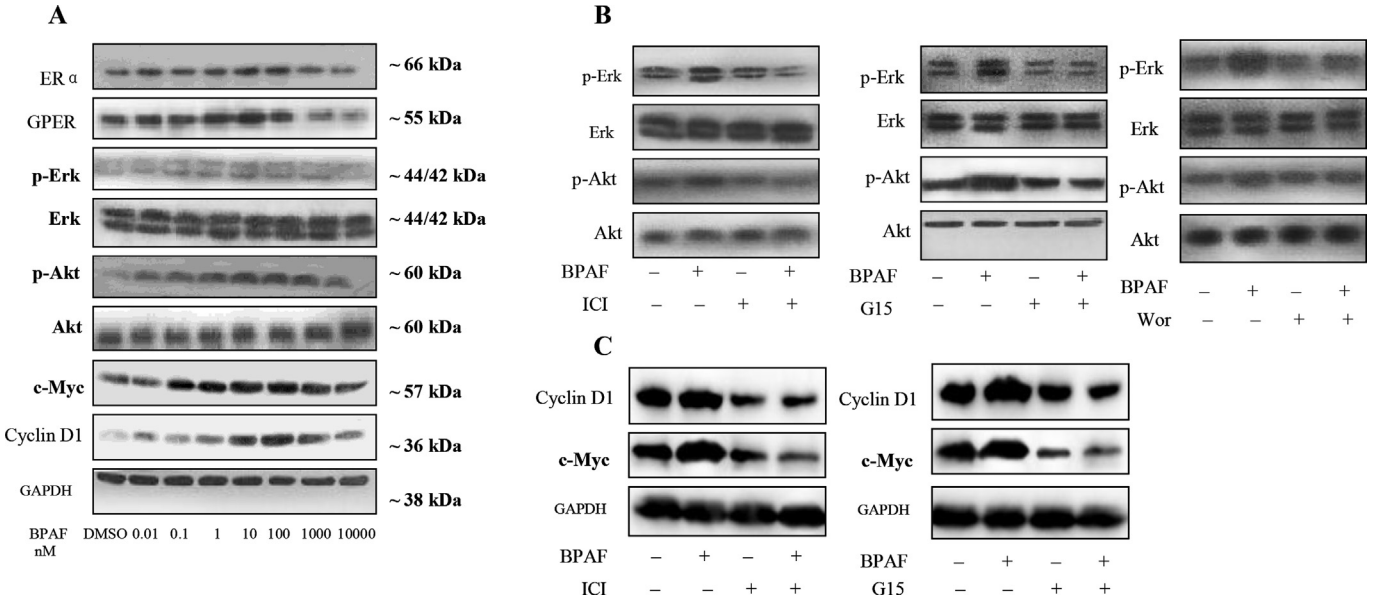
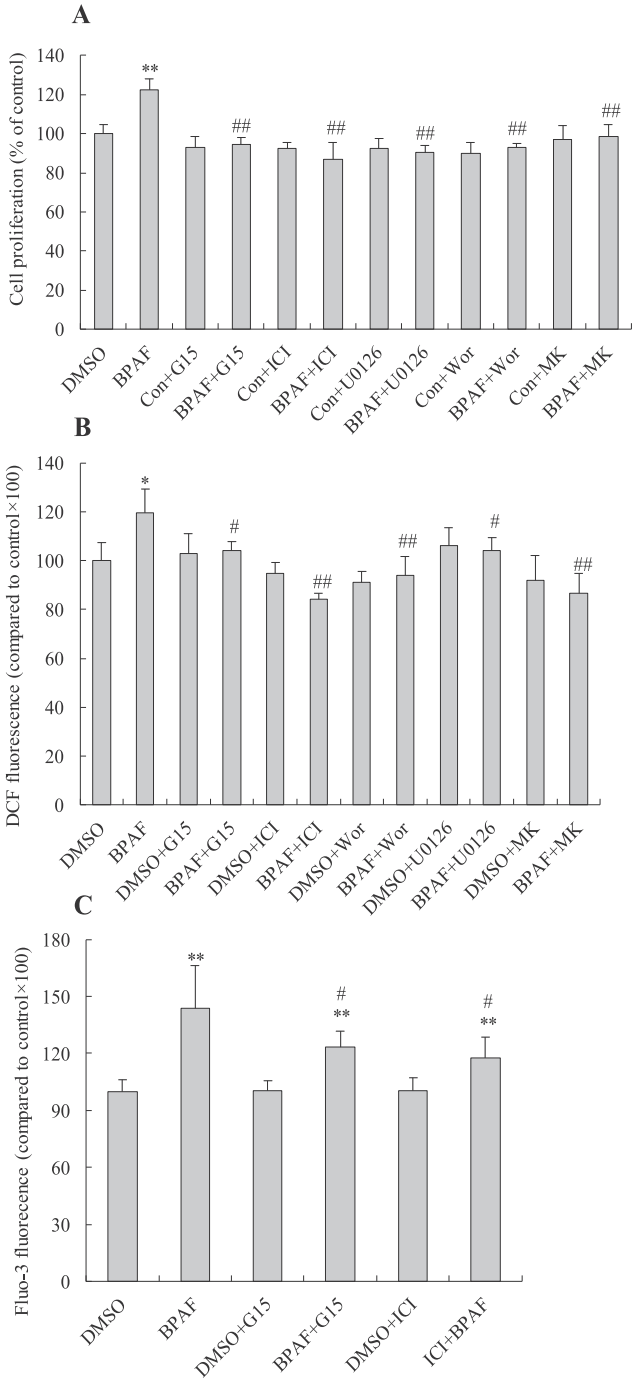


Fig. 4. Effects of BPAF (0.01–10000 nM) on the expression of GPER, ER*α*, *c*-Myc and Cyclin D1 and the phosphorylation of Erk and Akt by western blot analysis (A); effects of 10 nM BPAF on the phosphorylation of Erk and Akt with or without pre-treatment using specific signaling inhibitors for 1 h (B); effects of inhibitors ICI and G15 on the protein expression of *c*-Myc and Cyclin D1 (C).

2014; Tajbakhsh et al., 2018). *In vitro* research indicated that breast epithelium proliferation is dependent on the Ca^{2+} pathway via activation of mitogen-activated protein kinase (MAPK) by E2 (Tajbakhsh et al., 2018). Previous work found that BPAF increased levels of intracellular Ca^{2+} and ROS, and upregulated MAPK phosphorylation in mouse primary neuronal cells and hippocampal cells (Lee et al., 2013). In addition, E2 can rapidly modulate intracellular Ca^{2+} release via the ER-coupled signal transduction pathway (Muchekehu and Harvey, 2008). A recent report showed that BPAF elevates intracellular Ca^{2+} levels by activating GPER in SKBR3 cells (Cao et al., 2017). Therefore, the Ca^{2+} levels in MCF-7 cells ($ER\alpha^{+}$ and $GPER^{+}$) induced by BPAF may be regulated by ERα and GPER signals.



BPAF: 10 nM; ICI: 1 μM; G15: 10 μM; Wor: 10 nM; U0126: 10 nM; MK: 10 nM

Fig. 5. Effects of 10 nM BPAF on the cell proliferation (A), intracellular ROS (B), and Ca^{2+} (C) levels with or without pre-treatment using specific signaling inhibitors for 1 h. The values are expressed as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with DMSO; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with BPAF + ICI treatment.

3.4. Effects of BPAF on investigated protein expression

To further identify potential molecular mechanisms of BPAF induced cell biological responses such as cell proliferation, intracellular ROS and Ca^{2+} levels, ERα and GPER mediated Erk and PI3K/Akt pathways were used as main targets. The target proteins containing ERα, GPER, Erk, Akt, Cyclin D1, and *c*-Myc were analyzed by western blot. We found that BPAF upregulated the ERα, GPER, Cyclin D1, and *c*-Myc protein expression; increased the Akt and Erk phosphorylation in a concentration-dependent manner; and the expression of these proteins reached the maximum at 10 or 100 nM (Fig. 4A). Therefore, in the next experiment, 10 nM was used as an exposure concentration of BPAF to investigate the signal transduction pathways involved in activation of Erk and Akt using target inhibitors. After the addition of ICI, G15, and Wor which are the inhibitors of ERα, GPER, and PI3K, respectively, phosphorylation levels of Erk and Akt were both inhibited (Fig. 4B) and the numerical values of protein bands are shown in Fig. S6 and Fig. S7. Additionally, the addition of ICI and G15 also decreased the expression of *c*-Myc and Cyclin D1 proteins (Fig. 4C). Besides, the cell proliferation and intracellular ROS generation caused by BPAF were inhibited by ERα inhibitor ICI, GPER inhibitor G15, PI3K inhibitor Wor, Erk inhibitor U0126, and Akt inhibitor MK (Fig. 5A and B). Intracellular Ca^{2+} fluctuation were also attenuated by ICI and G15 (Fig. 5C). Furthermore, the phosphorylation of Akt and cell proliferation induced by BPAF were reduced by silencing GPER protein expression (Fig. 6B). The results further indicated that BPAF can activate the Akt signals via the GPER pathway, which mediated the cell proliferation induced by BPAF. These findings suggest that via GPER, BPAF can activate PI3K/Akt and Erk signals and that they, in turn, regulate the cell biological effects induced by low-concentrations of BPAF. Also, ERα plays critical roles in these BPAF induced cell biological responses.

In the present study, the down-regulation of GPER decreased phosphorylation levels of Erk and Akt, which was involved with attenuated MCF-7 cell proliferation. GPER is a new seven-transmembrane receptor. Estrogens through binding to or activation of GPER can rapidly activate downstream Erk and PI3K/Akt signals that facilitate breast cancer cell proliferation (Barton et al., 2018). Numerous studies also showed that GPER-regulated PI3K/Akt and Erk signals are critical regulators of estrogen-sensitive tumor cell proliferation induced by environmental estrogens (Du et al., 2012; Lei et al., 2017b, 2018a; Pupo et al., 2012). Our study indicated that GPER-mediated PI3K/Akt and Erk signals play a key role in MCF-7 cell proliferation induced by low-concentration BPAF.

The present study found that G15 and ICI of GPER and ERα inhibitors decrease the Cyclin D1 and *c*-Myc protein expression. Cyclin D1 is a member of the family of cyclin proteins, and *c*-Myc is a very strong proto-oncogene. Both of them are generally found to be upregulated in many types of cancer and are related to cell proliferation (Park et al., 2012; Pfeifer et al., 2015). These two proteins can be regulated via Ras/MAPK and PI3K (Deng et al., 2018; Chen et al., 2011, 2016). As reported in the literature, low-concentration BPA can induce cell proliferation by upregulating expression of *c*-Myc and Cyclin D1 proteins in mammary cells ($ERα^-$) (Pfeifer et al., 2015). In addition, bisphenol F (BPF) can induce MCF-7 cells to proliferate by enhancing expression of proteins such as Cyclin D1 and Cyclin E1 via the ERα-dependent pathway (Kim et al., 2017). All the results indicate that Cyclin D1 and *c*-Myc can also be regulated via GPER and ERα. Therefore, in the present case, *c*-Myc and Cyclin D1 may have important influences on cell viability or other cell biological effects induced by low concentrations of BPAF. It is possible that BPAF activates the signals of ERα and GPER-regulated Erk and PI3K/Akt, which activates downstream transcription of *c*-Myc and Cyclin D genes, ultimately resulting in the proliferation of MCF-7 cells. However, the specific mechanisms through which *c*-Myc and Cyclin D1 regulate proliferation or other biological effects in MCF-7 cells exposure to BPAF need further study.

Moreover, ERα inhibitor ICI decreased phosphorylation levels of Erk and Akt, which indicated that ERα has interaction with the GPER mediated PI3K/Akt and Erk signals. Besides, we observed that ERα and GPER interact directly by using ERα inhibitor ICI and GPER inhibitor G15 (Fig. S8). Similarly, Vivacqua et al. (2009) also found that ERα and GPER can directly interact in a coimmunoprecipitation experiment in the Ishikawa cells. The previous studies found that

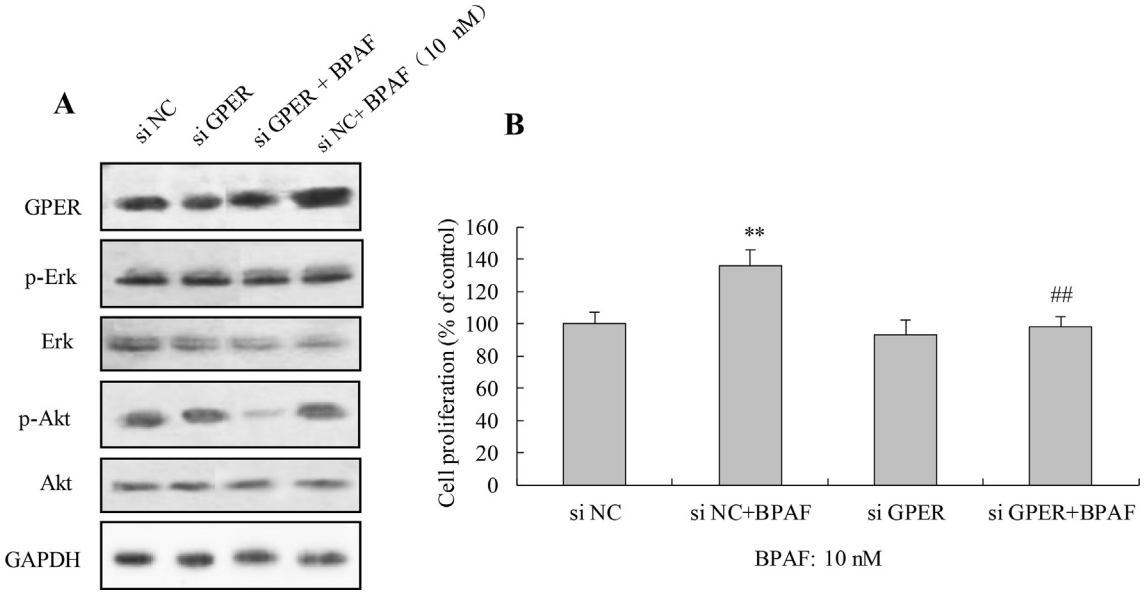


Fig. 6. Protein expression of GPER, *p*-Erk, and *p*-Akt with or without treatment with siGPER + BPAF (10 nM) (A) and cell proliferation after treatment with 10 nM BPAF for 24 h (B). NC, non-specific siRNA vector. Data points represent mean ± SD of three independent experiments. $^{**}p < 0.01$, compared to control (siNC); $^{\#\#}p < 0.01$, compared to treatment with siNC + BPAF.

estradiol or environmental estrogens such as atrazine, BPA, and thiodiphenol (TDP) can induce the proliferation of estrogen-sensitive cancer cells via crosstalk between nuclear ERα and membrane GPER-mediated signaling pathways (Albanito et al., 2008; Lei et al., 2017b; Romano and Gorelick, 2018; Sheng and Zhu, 2011; Silva et al., 2010; Wei et al., 2012; Zhang et al., 2017). These results indicated that BPAF can via crosstalk of ERα and GPER mediated signals to induce proliferation of MCF-7 cells. MCF-7 breast cancer cell proliferation induced by chemicals by activating ERα and GPER pathways can be used as an estrogenic property value of chemicals (Shim et al., 2014). Therefore, BPAF exhibits estrogenic property in MCF-7 cells.

In addition, intracellular ROS and Ca^{2+} levels induced in MCF-7 cells by BPAF were attenuated by specific signaling inhibitors. Estrogens can cause rapid increases in intracellular Ca^{2+} or ROS by activating or binding to GPER in estrogen-sensitive tumor cells and can induce them to proliferate (Barton et al., 2018; Lei et al., 2018b; Mahalingaiah and Singh, 2014; Tajbakhsh et al., 2018). The corresponding signal transduction includes an ER coupled to the PI3K/Akt and PKC/PKA/Erk pathways (Barton et al., 2018; Deng et al., 2018; Muchekehu and Harvey, 2008). Similarly, the present study also found that intracellular Ca^{2+} and ROS levels in MCF-7 cells induced by BPAF can be mediated by ERα or GPER-mediated PI3K/Akt and Erk signals and was possibly involved with cell viability.

4. Conclusions

BPAF in low concentrations induces significant effects on MCF-7 cells, including the promotion of cell proliferation and the elevation of intracellular ROS and Ca^{2+} levels. BPAF can activate GPER-mediated PI3K/Akt and Erk signals, while the activation of PI3K/Akt and Erk signals via GPER is one of mechanisms of the MCF-7 cell biological effects induced by BPAF. ERα also plays an important role in regulating these responses. The study of the cell biological response mechanism of BPAF via the GPER-mediated PI3K/Akt and Erk pathways deepened the understanding on the BPAF toxicity. The results indicated that as the substitute of BPA, the potential harm of BPAF to human health is also of concern. Taken together, these findings provide an important basis for the understanding of the differential biological activities and molecular mechanisms of BPAF that is ubiquitous in our everyday products.

Declaration of interest

We declare that there is no conflict of interest.

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Appendix A. Supplementary data

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