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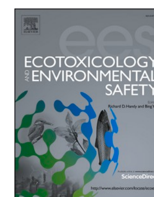

Fluorene-9-bisphenol regulates steroidogenic hormone synthesis in H295R cells through the AC/cAMP/PKA signaling pathway

Yuan Huang [a], Wei Zhang [a,*], Na Cui [a], Zhiming Xiao [a], Wenyu Zhao [a], Ruiguo Wang [a], John P. Giesy [b,c,d], Xiaoou Su [a,*]

[a] Institute of Quality Standards and Testing Technology for Agro-Products, Chinese Academy of Agricultural Sciences, Beijing 10081, China
[b] Department of Veterinary Biomedical Sciences, and Toxicology Center, University of Saskatchewan, 52 Campus Dr, Saskatoon, SK S7N 5B4, Canada
[c] Department of Integrative Biology and Center for Integrative Toxicology, Michigan State University, 784 Wilson Rd, East Lansing, MI 48824, USA
[d] Department of Environmental Science, Baylor University, 97266 One Bear Place, Waco, TX 76798, USA

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ABSTRACT

Fluorene-9-bisphenol (BHPF), which has been used as a substitute for bisphenol A (BPA) in consumer goods and industrial products, can be detected in environmental media and human urine. BHPF has been reported to have endocrine-disrupting effects, whereas deleterious effects on steroidogenesis in H295R cells and underlying mechanisms are still unclear. Here, we investigated effects of BHPF on steroidogenesis using human adrenocortical carcinoma cells (H295R). Cytotoxicity was initially assessed and half-maximal inhibitory concentration (IC_{50}) was determined based on proliferation of cells. Responses of four steroid hormones, aldosterone, cortisol, testosterone and 17β-estradiol (E_2), and ten critical genes, *StAR*, *HMGR*, *CYP11A1*, *CYP11B1*, *CYP11B1*, *HSD3B2*, *CYP21*, *CYP17*, *17β-HSD*, and *CYP19,* involved in steroidogenesis after exposure to non-cytotoxic concentrations of BHPF were determined in the presence or absence of 100 μM dbcAMP. Adenylate cyclase (AC) activity, intracellular concentrations of cAMP, PKA activity and amounts of steroidogenic factor-1 (SF-1) gene and expressions of proteins were determined to elucidate underlying mechanisms of effects on steroidogenesis. BHPF was cytotoxic to H295R cells in a dose- and time-dependent manner. Effects on production of hormones results demonstrated that exposure to greater concentrations of BHPF inhibited productions of aldosterone, cortisol, testosterone and E_2 by down-regulation of steroidogenic genes. Inhibition of AC activity, intercellular cAMP content and PKA activity after exposure to BHPF implied that the AC/cAMP/PKA signaling pathway was involved in BHPF-induced suppression of steroidogenesis in H295R cells. Additionally, BHPF inhibited steroidogenesis and expressions of steroidogenic genes via decreasing expression of SF-1 protein, both in basal and dbcAMP-induced treatment. These results contributed to understanding molecular mechanisms of BHPF-induced effects on steroidogenesis and advancing the comprehensive risk assessment of BPs.

1. Introduction

Bisphenols are widely produced for the production of polycarbonate (PC), epoxy resins, and polyurethane, which are used in numerous daily necessities, including paper cups, can coatings, plastic products, thermal paper, and electronics (Karrer et al., 2019; Xue et al., 2019). Humans are frequently exposed to bisphenols (BPs) in daily life, which have adverse effects on human health, especially the endocrine-disrupting effects.

Fluorene-9-bisphenol (BHPF) is one of the alternatives to bisphenol-A (BPA) and can also be used to produce polymer materials (Liu et al., 2008; Zhen et al., 2009), which are applied to produce protective coatings, insulation materials, and adhesives as well as daily necessities such as bottles for drinking water, takeaway packaging, and coatings of metal cans. BHPF can be detected in natural water sources (Jin and Zhu, 2016). BHPF has been found in imported PC plastic bottles and their bottled water and BPA-free bottled water (Zhang et al., 2017). BHPF is also detected in the blood serum of college students with a mean concentration of 0.34 ± 0.21 ng/mL (Zhang et al., 2017), which is greater than that of the substitutes for BPA, bisphenol-F (BPF) and bisphenol-S (BPS), which have mean concentrations of 0.087 ng/mL and 0.0089

* Corresponding authors.
E-mail addresses: Huangyuan_171@126.com (Y. Huang), zhangwei03@caas.cn (W. Zhang), cuina0423@163.com (N. Cui), xiaozhiming@caas.cn (Z. Xiao), 17864272808@163.com (W. Zhao), wangruiguo@caas.cn (R. Wang), jgiesy@aol.com (J.P. Giesy), suxiaoou@caas.cn (X. Su).

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ng/mL, respectively, in blood serum of people living near e-waste stations (Song et al., 2019).

Recently, results of studies have demonstrated potential hazards of BHPF to the health of animals or humans. BHPF has been reported to be an anti-estrogen, which caused reduced mass of the uterus, induced endometrial atrophy and adversely affected pregnancy in mice (Zhang et al., 2017). In the reporter gene bioassays for detecting activities of BHPF against steroid hormone receptors, BHPF was found to be a potent antagonist for ER but not for androgen (AR), progesterone (PR), glucocorticoid (GR) or mineralocorticoid (MR) (Grimaldi et al., 2019). IC_{50} values based on the antagonism of BHPF as determined using the dual-luciferase report test, to ERα and ERβ are 109 nM and 75.3 nM, respectively (Zhang et al., 2017). These results further indicate that BHPF can exert anti-estrogen effects via competitively binding to the ER. BHPF not only interferes with the endocrine system but also has adverse effects on reproduction and potential behaviors (Mi et al., 2019). BHPF has toxic effects on the ovaries of different species due to inhibition of maturation and development of oocytes, and reduced quality of oocytes due to damage to the cytoskeleton, oxidative stress, damage of DNA, mitochondrial dysfunction, and cell apoptosis (Jia et al., 2019; Jiao et al., 2019, 2020). BHPF can interfere with male and female zebrafish's courtship preferences, induce anxiety and depression-like symptoms, and down-regulate the expression of estrogen receptors (*esr1* and *esr2a*) and steroid hormone synthesis-related genes (*hsd3b*, *cyp17a1* and *cyp19a1a*) (Mi et al., 2019).

Steroid hormones are a class of tetracyclic aliphatic, hydrocarbon compounds produced in steroid-synthesizing cells in adrenal glands, ovaries, testes, and brain. The adrenal gland, which is responsible for the production of three major types of steroid hormones: mineralocorticoids, glucocorticoids, and sex hormones is one of the most important endocrine organs for maintaining homeostasis in humans (Li et al., 2013). It has been reported that the effects of BPA, BPS, BPF, and BPAF on the steroidogenesis and steroidogenic gene expressions in H295R cells (Zhang et al., 2011; Feng et al., 2016), but the effects of BHPF on steroidogenesis are still unknown. BHPF has been found to down-regulate some steroidogenic gene expression on zebrafishes (Mi et al., 2019), mechanisms involved in the mediation of steroidogenesis are still lacking.

To elucidate the effects of BHPF and underlying mechanisms of effects of BHPF on steroidogenesis, human adrenocortical adenocarcinoma cell line (H295R) was used to measure secretion levels of steroid hormones as well as expressions of enzymes and genes in the steroidogenic signaling pathway. H295R cell line is currently an ideal model for studying steroid hormone synthesis since it retains all steroidogenic pathways and secretes all steroid intermediates of steroidogenesis (Gazda et al., 1990; Hecker et al., 2006; Hecker and Giesy, 2008). In this study. Cytotoxicity and effects on the secretion of steroid hormones were analyzed in H295R cells after exposure to BHPF, and the role of the AC/cAMP/PKA signaling pathway in modulating adverse effects of BHPF was investigated.

2. Materials and methods

2.1. Chemicals

Fluorene-9-bisphenol (BHPF, CAS no. 3236–71–3, 97.5% purity) was purchased from Dr. Ehrenstorfer Inc. (Augsburg, Germany). Dibutyryl-cAMP sodium salt (dbcAmp, CAS no. 16980–89–5, 99.71% purity) and forskolin (FSK, CAS no. 66575–29–9, 99.82% purity) were obtained from MedChemExpress Co. Ltd. (Monmouth Junction, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St.Louis, USA). All drugs were dissolved in DMSO to form the stock solutions.

2.2. Cell culture

H295R cell line was obtained from Cell Bank (China Academy of Medical Sciences, Institute of Basic Medical Center of Cell). Cells were cultured in 75-cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Gibco, Grand Island, USA) supplemented with 1% insulin-transferrin-selenium-G (ITS-G, Gibco, Grand Island, USA), 5.35 μg/mL linoleic acid (Sigma, USA), 1.25 mg/mL bovine serum albumin (BSA, Sigma, USA), 1% penicillin-streptomycin (Gibco, USA) and 2% Ultroser G (Pall, Port Washington, USA) at 37 ℃ in a humidified atmosphere containing 5% CO_2. The exposure medium consisted of phenol red-free DMEM/F12 medium supplemented with 1% ITS-G, 1% penicillin-streptomycin, and 10% charcoal-stripped FBS. BHPF dissolved in DMSO were added to the medium after cells adhered for 24 h. The final concentration of DMSO in the culture medium was $\leq$ 0.1% (v/v). Control cells were incubated with 0.1% DMSO to match the final concentration achieved in culture medium in the experimental exposures.

2.3. Cell viability assay

In the cell viability assay, H295R cells were seeded into 96-well microplates with a density of 3×10^4 cells/well. Cell counting kit-8 (CCK-8, Dojindo, Japan) assay was utilized to detect the cell viability of H295R cells exposed to BHPF. Cells were treated with BHPF (0, 10, 20, 30, 40, 50, 60, 70 or 100 μM) alone for 24 h, 48 h and 72 h, or combined with 100 μM dbcAMP for 48 h. For each duration of exposure, the CCK-8 reagent was added to the culture medium and incubated for 3 h at 37 ℃. Absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA), and the 650 nm was set as the reference wavelength to deduct the background interference caused by the turbid media. The assay was conducted in triplicate with six replicates of each concentration. The relative cell viability was presented as a percentage of treatment groups compared to the DMSO control group, which are subtracted from the absorbance of the background control.

2.4. Cell proliferation assay

H295R cells were grown in 96-well microplates with a density of 2×10^4 cells/well for 24 h to adhere. Proliferation of H295R cells exposed to 0, 10, 20, 30, 40 and 50 μM BHPF for 48 h was assessed by a BeyoClick EdU-555 cell proliferation assay kit (Beyotime, Shanghai, China). According to the instruction of the kit, 10 mM EdU was diluted to 5 μM from fresh cell medium to prepare the EdU working solution. The BHPF-containing cell culture medium was removed and the fresh EdU working solution was added in 100 μL/well and incubated at 37 ℃ in the dark for 2 h. H295R cells were fixed with 4% paraformaldehyde for 15 min, washed three times and permeabilized with 0.3% Triton X-100 for 15 min. H295R cells were washed twice and incubated with click reaction buffer in the dark for 30 min at room temperature. Finally, cells were counterstained with 1 × Hoechst 33342 (10 μM) and incubated in the dark for 10 min. After being washed three times, cells were observed and the images were analyzed with a high-content screening system (PerkinElmer, USA).

2.5. Hormone measurement

H295R cells were cultured in DMEM/F-12 without phenol-red and seeded into 24-well plates at a density of 2.5×10^5 cells/well. After 24 h of pre-culture to adhere completely, cells were exposed to 0, 0.01, 0.1, 1, 10, 20 or 30 μM BHPF in the basal treatment or 100 μM dbcAMP-induced treatment, respectively. The DMSO group was the control of the basal treatment, and the dbcAMP-induced group was the control of the dbcAMP-induced treatment. After exposure for 48 h, cell culture medium in each well was collected and stored at − 20 ℃ for hormone measurement. Concentrations of aldosterone, cortisol, testosterone (T) and 17β-estradiol (E_2) were determined, using commercially available radioimmunoassay kits from Beijing North Institute of Biological Technology according to the manufacturer's instructions. Sensitivities of

each RIA kit was 0.02 ng/mL (for aldosterone), 2 ng/mL (for cortisol), 0.02 ng/mL (for T) and 5 pg/mL (for E_2), respectively. The intra-assay and inter-assay coefficients of variation were less than 10% and 15%, respectively.

2.6. RNA extraction and RT-qPCR

H295R cells were grown in 6-well cell culture plates with a density of 1×10^6 cells/well. When the cell confluence reached approximately 70%, H295R cells were exposed to 0, 0.01, 0.1, 1, 10, 20 and 30 μM BHPF in the presence or absence of 100 μM dbcAMP for 48 h. Total RNA was isolated from the cells by use of the RNAprep Pure Cell Kit (TIANGEN, Beijing, China) following the manufacturer's instruction. Concentrations of total RNA were measured at 260 nm using NanoDrop (Thermo Scientific, USA). All RNA samples exerted suitable A260/A280 and 28 S/18 S ratios. Reverse transcription of RNA and PCR amplification was performed using the One Step TB Green PrimeScript RT-PCR Kit (Takara, Japan) with QS5 real-time PCR system (Applied Biosystem Inc., USA). PCR primers were listed in Table S1. Target gene (*StAR*, *HMGCR*, *CYP11A1*, *CYP11B1*, *CYP11B1*, *CYP19*, *CYP17*, *CYP21*, *17β-HSD*, *HSD3B2, SF-1*) expression levels were normalized to *β*-actin expression values and calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). The amplification efficiencies of primers varied in the range of 93.3%− 104.2%, which has met the criteria for high amplification efficiency of RT-qPCR.

2.7. Quantification of AC activity and intracellular cAMP

H295R cells were seeded into T25 cell culture flask at a density of 3 $\times 10^6$ cells/well and cultured for 24 h. After the exposure of 30 μM BHPF or in the presence of 10 μM FSK to H295R cells for 48 h, the adenylate cyclase (AC) activity was determined by the amount of cAMP produced. Isolation of cell membrane proteins was performed using the CelLytic MEM Protein Extraction Kit (Sigma, USA) by two steps according to the manufacturer's instruction, including cell collection and separation of hydrophobic and hydrophillic proteins. The lower hydrophobic phase enriched with hydrophobic and raft associated proteins was retained for measurement of AC activity according to the method described previously (Wang et al., 2015). Membrane protein (30 μg) was added to the reaction buffer (50 mM Tris-HCl, pH 7.4; 5.0 mM $MgCl_2$; 0.5 mM EDTA; 1 mM ATP; 0.1 mM GTP; 0.2 IU pyruvate kinase; 0.1 unit of myokinase and 2.5 mM phosphoenolpyruvate), and incubated at 37 °C for 15 min. The converted cAMP content was detected by enzyme-linked immunosorbent assay (ELISA) kit (Cayman, Michigan, USA).

Intracellular concentrations of cAMP were determined by use of the cAMP ELISA kit based on the manufacturer's instructions. Absorbance was measured at 410 nm by a microplate reader, and cAMP content was calculated in accordance with the standard curve.

2.8. PKA activity assay

H295R cells were exposed for 48 h to 30 μM BHPF in the presence or absence of 100 μM dbcAMP or 10 μM FSK. Cell lysates were obtained by sonication in an ice bath, and PKA activity was analyzed using ELISA kits (Invitrogen, Frederick, USA) according to the manufacturer's instruction. In brief, the appropriate samples and reconstituted adenosine triphosphate (ATP) were added into each well of the microtiter plate and incubated for 90 min at 30 ℃ with shaking. Then, the detection antibody was added and incubated for 60 min at room temperature. The chromogen was finally added and incubated for 30 min at room temperature. After adding the stop solution, the absorbance was measured at 450 nm.

2.9. Western blotting

Total protein was extracted from cells using RIPA lysis buffer (Beyotime, Shanghai, China) with a protease inhibitor PMSF and a phosphatase inhibitor (Beyotime, Shanghai, China) according to the manufacturer's instructions. Concentrations of protein were determined by a BCA Protein Assay Kit (TIANGEN, Beijing, China). Protein samples (30 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted onto a polyvinylidene difluoride (PVDF) membrane. Subsequently, membrane was blocked in 5% skimmed milk and incubated overnight at 4 ℃ with primary antibodies against phospho-PKA substrate (#9624 S, CST, USA), SF-1 (#12800 S, CST, USA) and *β*-actin (#4970 S, CST, USA). After washing three times with TBST, the PVDF membrane was incubated with the anti-rabbit IgG-HRP antibody (#7074 S, CST, USA) for 1 h at room temperature. Immunoblots were prepared with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo, USA), and detected by Tanon 5200 chemiluminescence/fluorescence image analysis system (Tanon, Shanghai, Beijing). The binds intensity was quantified using ImageJ software (LiCor, Lincoln, NE, USA). Relative expression of each protein was calculated by normalization to *β*-actin, and the resulting ratios in the control group were normalized to 1.

2.10. Statistical analysis

The analysis of hormone production and gene transcription profiles was processed with SPSS 25.0 for windows (SPSS Inc., Chicago, USA). A Shapiro-Wilks normality test indicated that parameters met the assumption of normal distribution required for the application of parametric statistical inferences. The assumption of homogeneity of variance was assessed using Levene's test. The statistical significance of differences was calculated using one-way analysis of variance (ANOVA) followed by post hoc LSD tests. A value of $p < 0.05$ was considered statistically significant. The IC_{50} values of the cytotoxic of BHPF and all result graphs were performed using GraphPad Prism 9.0 (GraphPad Software Inc. San Diego, USA). The results were represented as mean with standard deviation (SD) in triplicate within three independent experiments.

3. Results

3.1. Effects of BHPF on cell viability and proliferation

The IC_{50} values for H295R cells exposed to BHPF for 24 h, 48 h, 72 h were 48.82 μM, 44.28 μM and 40.28 μM, respectively. These results indicated that BHPF was cytotoxic to H295R cells in a dose- and time-dependent manner (Fig. 1 A). Meanwhile, no significant cytotoxicity on H295R cells of BHPF were observed at concentrations of 0.01, 0.1, 1, 10, 20 and 30 μM in the presence of 100 μM dbcAMP (Fig. 1B). However, significant cytotoxicity was observed in cells exposed to 40 μM BHPF($P < 0.01$). In addition, 40 μM BHPF significantly inhibited DNA synthesis of H295R cells in the BeyoClick EdU-555 cell proliferation assay ($P < 0.01$), exerting significant cytotoxicity on the cell proliferation (Fig. 1 C and Fig. 1D). Thus, concentrations of 0.01, 0.1, 1, 10, 20 and 30 μM BHPF were selected for further research.

3.2. Effects of BHPF on the production of steroid hormones

Effects of BHPF (0.01–30 μM) on the steroid hormones produced by H295R cells were investigated in the presence or absence of dbcAMP (Fig. 2). Compared to the DMSO control, levels of aldosterone and cortisol were not significantly altered at tested concentrations ranging from 0.01 to 10 μM. However, aldosterone production decreased to 77% and 36%, and cortisol production decreased to 71% and 35% after 20 μM and 30 μM BHPF exposure, respectively (Fig. 2 A and Fig. 2B). In dbcAMP-induced exposure, the production of aldosterone and cortisol was significantly increased ($P < 0.01$), whereas they were inhibited after exposure to 20 μM or 30 μM BHPF. Compared to the dbcAMP control, aldosterone production was reduced to 76% and 78%, and cortisol production was reduced to 53% and 14% (Fig. 2 A and Fig. 2B).

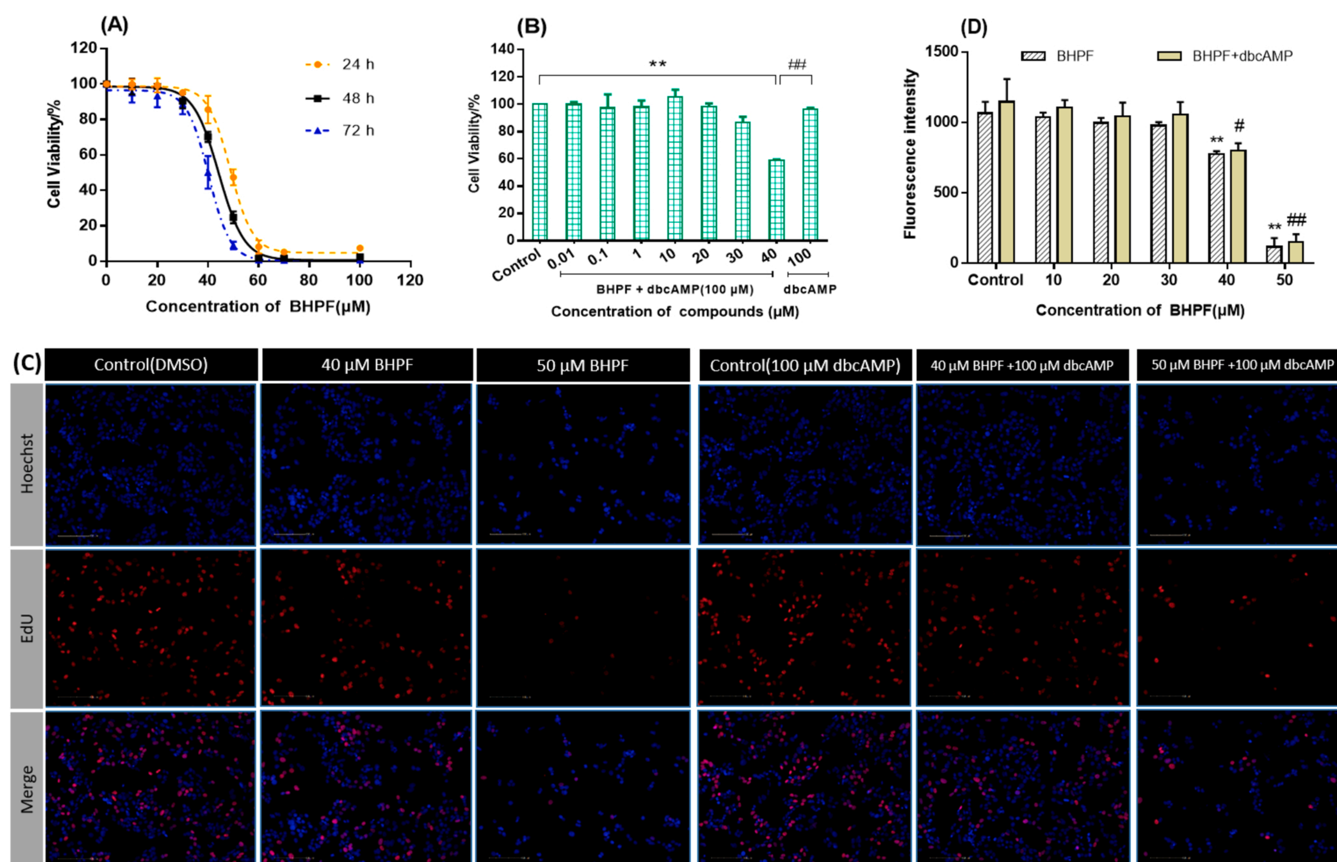

Fig. 1. Cytotoxicity of BHPF on H295R cells. (A) Cell viability of H295R cells exposed to 0–100 μM BHPF for 24 h (orange), 48 h (black), and 72 h (blue); (B) Cell viability of H295R cells exposed to 0–40 μM BHPF in the presence of 100 μM dbcAMP for 48 h; (C) Micrographs presenting the incorporation of EdU in H295R cells exposed to BHPF or combined with 100 μM dbcAMP for 48 h. Red: the EdU positive cells; blue: the cell nuclei (Hoechst); (D) Cell proliferation level was quantitatively analyzed by the percentage of EdU positive cells versus total cell numbers. Results are means ± SD of three independent experiments conducted in 6 wells per exposure dose. Compared to the DMSO control, * $P < 0.05$, * * $P < 0.01$; Compared to the dbcAMP-induced control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

No statistically significant changes were observed for aldosterone and cortisol production in cells exposed to 0.01–10 μM BHPF (Fig. 2 A and Fig. 2B).

Compared to the DMSO control cells, the production of testosterone increased to 1.40-fold in cells exposed to 0.01 μM BHPF, whereas it declined at greater concentrations of BHPF in a dose-dependent manner (Fig. 2 C). A significant reduction in testosterone production was observed at 10, 20 and 30 μM BHPF to 31%, 31% and 14%, respectively (Fig. 2 C). Exposure to 0.01 μM BHPF increased testosterone production to 1.24-fold relative to the dbcAMP-induced control, while testosterone production was reduced to 74% in cells exposed to 30 μM BHPF (Fig. 2 C). The production of E_2 was 1.33-fold greater in cells exposed to 1 μM BHPF than that of the DMSO control but decreased to 38% in cells exposed to 30 μM BHPF (Fig. 2D). In the presence of dbcAMP, no significant effects on E_2 production were observed in cells exposed to concentrations of BHPF from 0.01 to 20 μM (Fig. 2D). Nevertheless, the production of E_2 significantly decreased to 57% in cells exposed to 30 μM BHPF (Fig. 2D).

3.3. Effects of BHPF on the transcription of steroidogenic genes

Transcriptions of critical genes encoding enzymes involved in steroidogenesis, including HMGR, StAR, CYP11A1, CYP11B1, CYP11B1, CYP19, CYP17, CYP21, 17β-HSD and HSD3B2, were affected by exposure to BHPF (Fig. 3). Compared to the DMSO control, expression of *HMGR* was down-regulated to 83%, 87%, 86% and 76% in cells exposed to concentrations of BHPF from 1 to 30 μM, and to 82% and 62% of the dbcAMP-induced control in cells exposed to 20 μM or 30 μM BHPF (Fig. 3A). Expression of *StAR* was down-regulated to 87%, 82% and 66% relative to the DMSO control in cells exposed to 1, 20 and 30 μM BHPF, while *StAR* was downregulated to 66% of the dbcAMP-induced control when cells were exposed to 30 μM BHPF (Fig. 3B). Expression of *CYP11A1* was down-regulated to 78–89% of the DMSO control in cells exposed to 0.1–30 μM BHPF, and to 80% and 66% of the dbcAMP-induced control when exposed to 20 μM and 30 μM BHPF, respectively (Fig. 3C). Expression of *HSD3B2* was downregulated to 80% and 42% of the DMSO control, and 72% and 45% of the dbcAMP-induced control when cells were exposed to 20 μM and 30 μM BHPF, respectively (Fig. 3D).

Expression of *CYP21A2* was not significantly altered by exposure to 0.1–30 μM BHPF, whereas exposure to 1–20 μM BHPF down-regulated expression of *CYP21A2* to 73–83% relative to the dbcAMP-induced control (Fig. 3E). Expression of *CYP11B1* was significantly down-regulated to 72% and 41% of the DMSO and dbcAMP-induced control in cells exposed to 30 μM BHPF (Fig. 3F). Exposure to 30 μM BHPF also down-regulated *CYP11B2* to 25% and 36% of the DMSO and dbcAMP-induced control (Fig. 3G). When H295R cells were exposed to 30 μM BHPF, *CYP17A1* was down-regulated to 73% and 63% of the DMSO and dbcAMP-induced control (Fig. 3H). In the basal treatment, 0.1 μM BHPF up-regulated *17βHSD* to 1.12-fold, while 20 μM and 30 μM BHPF down-regulated *17βHSD* to 82% and 84%. In dbcAMP-induced cells, concentrations in the range of 0.01–1 μM BHPF significantly up-regulated expression of *17βHSD* to 1.25-fold, 1.14-fold and 1.15-fold (Fig. 3I). Exposure to 0.01–30 μM BHPF down-regulated *CYP19A1* to 49–80% of the DMSO control and 44–87% of the dbcAMP control in a dose-dependent manner (Fig. 3J).

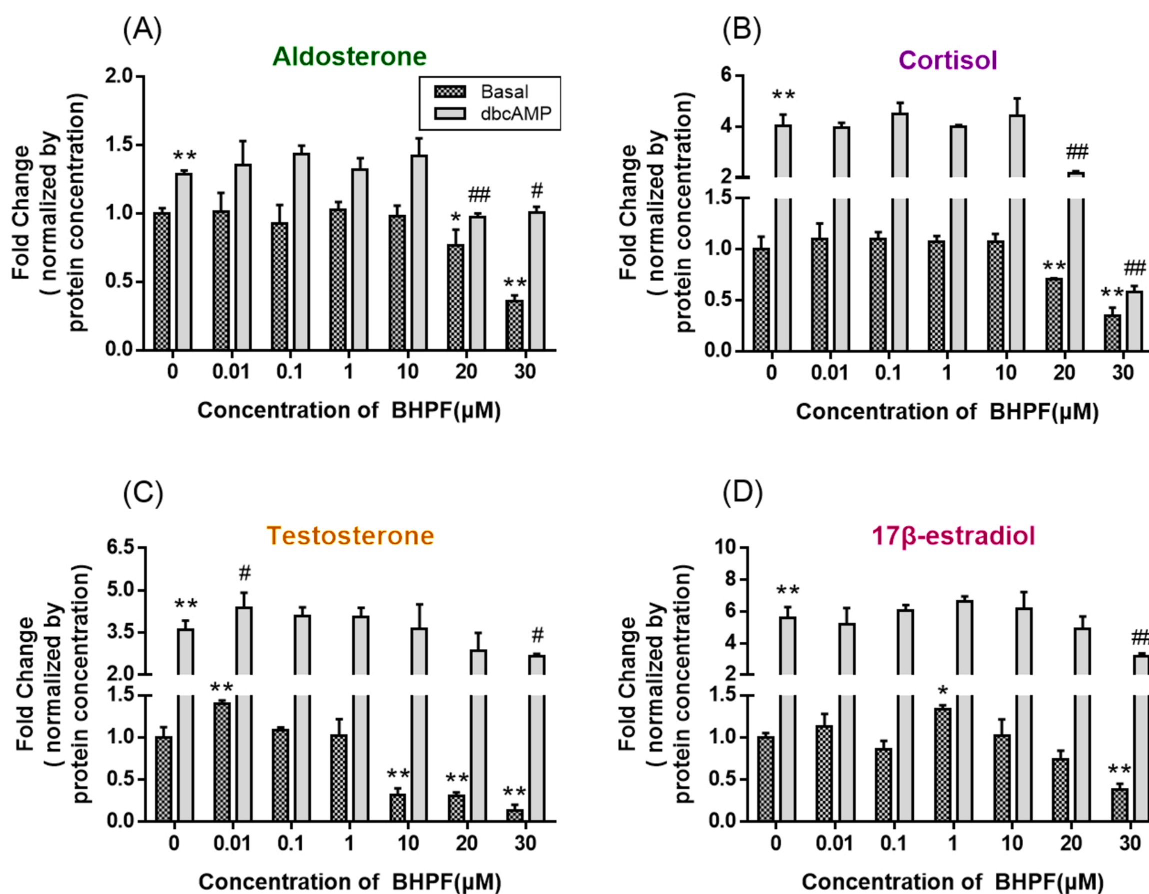

Fig. 2. Effects of BHPF on production levels of steroid hormones in the basal treatment and cAMP-induced treatment. H295R cells were exposed to 0–30 μM BHPF or in the presence of 100 μM dbcAMP for 48 h. Levels of steroid hormones were measured by RIA kits and fold changes were analyzed compared to the DMSO control group after normalization with protein concentration. (A) the fold change of aldosterone level; (B) the fold change of cortisol level; (C) the fold change of testosterone level; (D) the fold of 17β-estradiol level. Data are means ± SD of three replicate samples. Compared to the DMSO control, * $p < 0.05$ and * * $p < 0.01$; compared to the dbcAMP-induced control, $^{\#}p < 0.05$ and $^{\#\#}$ $p < 0.01$.

3.4. Effects of BHPF on AC activity and intracellular cAMP content

FSK is used to increase the level of intracellular cAMP via directly activating AC. The second-messenger cAMP plays a pivotal role in regulating steroidogenesis and other diverse cell functions. To further investigate the mechanism of inhibition on steroidogenesis of BHPF, we analyzed intracellular cAMP content and AC activity in H295R cells treated with BHPF alone or combined with FSK. As is shown in Fig. 4, 10 μM FSK increased AC activity and the level of intracellular cAMP to 1.56-fold and 2.86-fold of the DMSO control. When H295R cells were exposed to 30 μM BHPF, a declining trend of AC activity and cAMP content was observed, but no statistical differences compared to the DMSO control group. However, BHPF significantly inhibited AC activity to 38.4% and decreased cAMP content to 71.9% in the presence of FSK, compared to the FSK-induced control.

3.5. Effects of BHPF on PKA activity

One of the most important target proteins in the downstream signaling pathway of cAMP is PKA. The various physiological functions of cAMP are to activate PKA to phosphorylate its substrates, thereby regulating the expression of various key downstream factors. In order to further clarify the regulatory role of the cAMP/PKA signaling pathway in the inhibition of steroid hormone synthesis by BHPF, the effects of BHPF in the basal, FSK -induced and dbcAMP-induced treatments on PKA activity were examined by use of ELISA, and the phosphorylation substrate of PKA was analyzed by western blotting. Both 100 μM dbcAMP and 10 μ M FSK can activate PKA to 1.45-fold and 1.57-fold of the DMSO control, whereas PKA activity was suppressed to 76%, 78% and 67% of the corresponding control after exposure to 30 μM BHPF in the basal, dbcAMP-induced or FSK-induced treatments (Fig. 5).

3.6. Effects of BHPF on SF-1 expression

SF-1 is a critical regulator in steroid hormone synthesis. In order to further confirm the involvement of SF-1 in the BHPF-suppressed steroidogenesis, the alteration of gene and protein expressions of SF-1 was investigated (Fig. 6). In the basal treatment, 30 μM BHPF up-regulated expression of *SF-1* to 1.70-fold relative to the DMSO control, whereas down-regulated the protein expression of SF-1–62%. In the dbcAMP-induced treatment, dbcAMP significantly up-regulated the gene and protein expressions of SF-1–2.12-fold and 1.69-fold of the DMSO control, while 30 μM BHPF inhibited both gene and protein expression of SF-1–78% and 66% of the dbcAMP-induced control.

4. Discussion

The dose- and time-dependent cytotoxicity of BHPF to H295R cells observed in this study was consistent with similar effects of BPA, BPF, BPS, and BPAF, all of which have been reported to cause cytotoxicity to H295R cells (Feng et al., 2016). Based on the lesser IC_{50} for BHPF, it was more potent than BPA and its other analogues. The response concentration of BHPF might vary in different cell types. After human endometrial cancer Ishikawa cells were exposed to 50 μM BHPF for 24 h, the

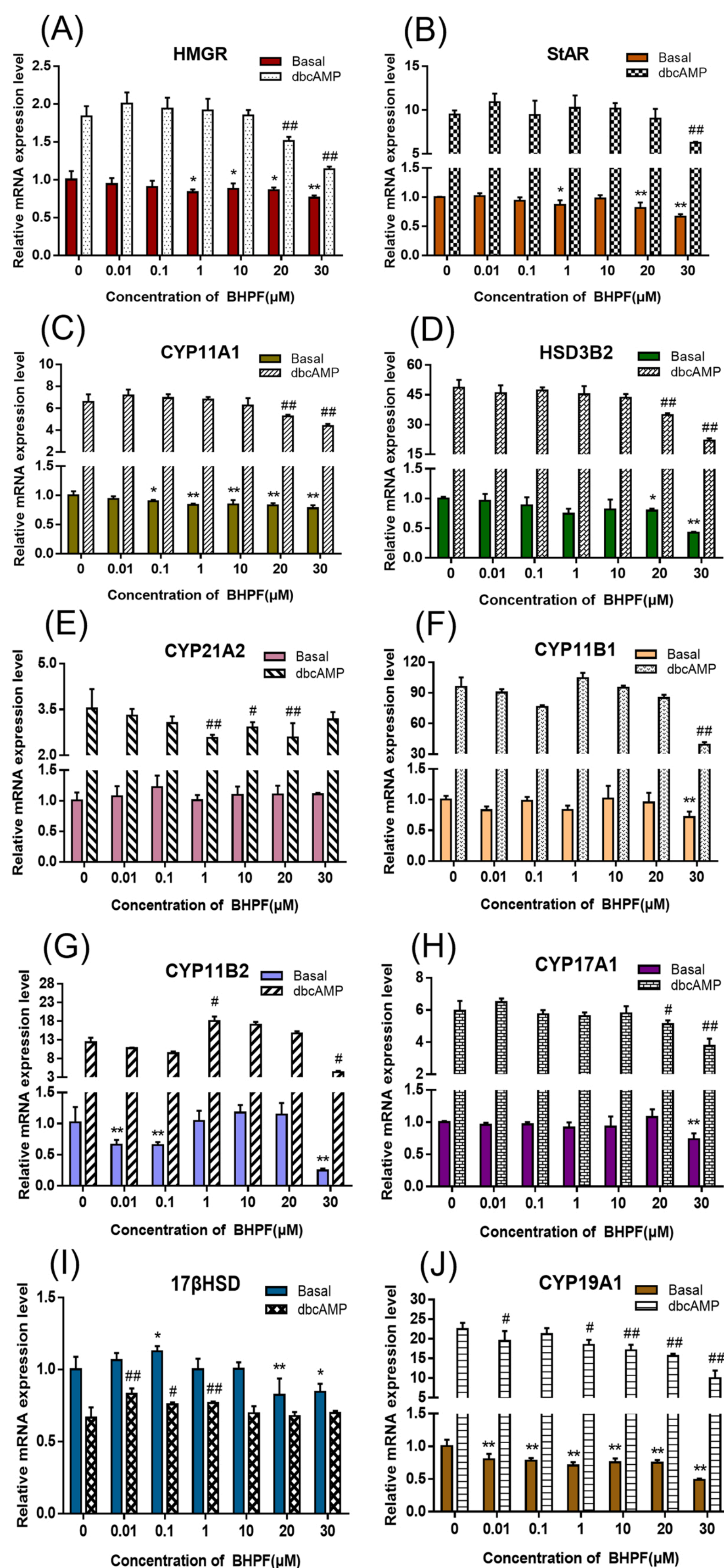

Fig. 3. Effects of BHPF on critical steroidogenic genes expression in the basal treatment and cAMP-induced treatment. H295R cells were exposed to BHPF in the absence or presence of 100 μM dbcAMP for 48 h. Results are means ± SD of three replicate samples. Compared to the DMSO control, * $p < 0.05$ and * * $p < 0.01$; compared to the dbcAMP-induced control, $^{\#}p < 0.05$ and $^{\#\#}$ $p < 0.01$.

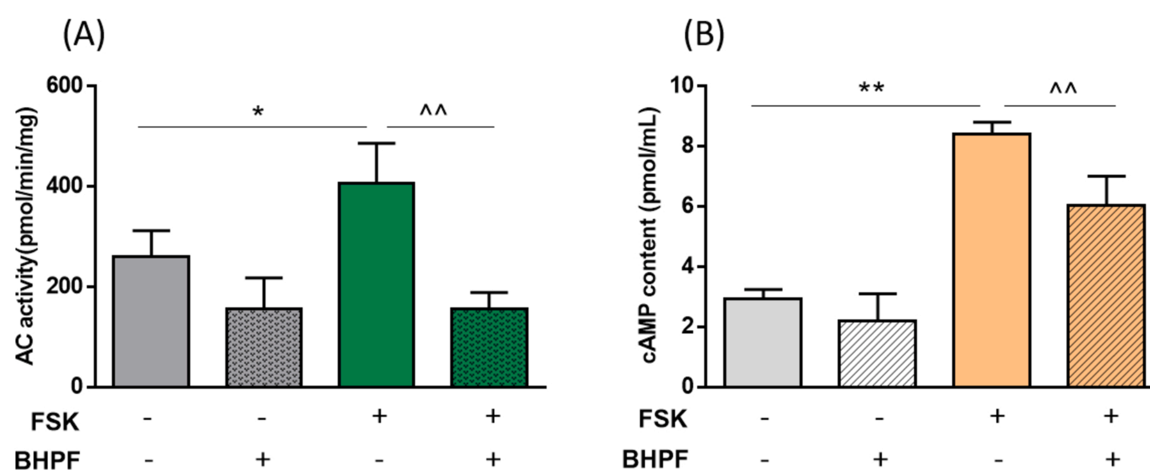

Fig. 4. Effects of BHPF on AC activity (A) and intracellular cAMP content (B). H295R cells were treated with 30 μM BHPF in the absence or presence of 10 μM forskolin (FSK) for 48 h. Results are means ± SD of three replicate samples. Compared to the DMSO control, * $p < 0.05$ and * * $p < 0.01$; compared to the FSK-induced control, $\hat{}$ $p < 0.05$ and $\hat{}\hat{}$ $p < 0.01$.

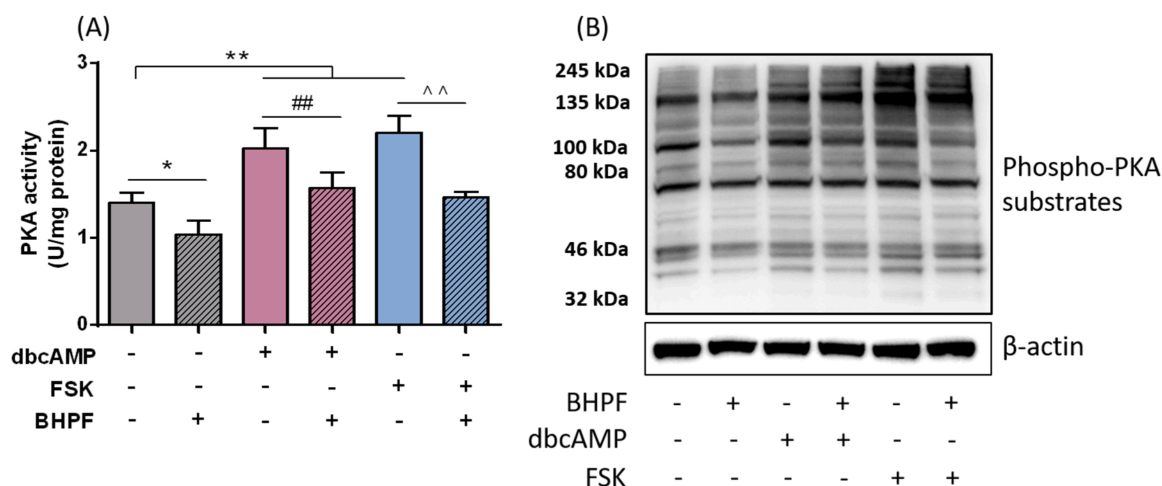

Fig. 5. Effects of BHPF on PKA activity. H295R cells were treated with 30 μM BHPF in the absence or presence of 10 μM forskolin (FSK) or 100 μM dbcAMP for 48 h. Results are means ± SD of three replicate samples. Compared to the DMSO control, * $p < 0.05$ and * * $p < 0.01$; compared to the dbcAMP-induced control, $^{\#}p < 0.05$ and $^{\#\#}$ $p < 0.01$; compared to the FSK-induced control, $\hat{}$ $p < 0.05$ and $\hat{}\hat{}$ $p < 0.01$.

cell viability was reduced to about 50%. However, no significant change in cell viability was observed with concentrations of BHPF increasing to 100 μM (Wang et al., 2019). In our work, the IC_{50} value (48.8 μM) of BHPF at 24 h was at the level comparable with the above, but the cell viability of H295R cells was significantly decreased to 8.3% and 7.6% of the control group after 60 μM and 100 μM BHPF exposure. In contrast, no significant effect on cell viability of the porcine Sertoli cells and meiosis in mouse oocytes were found after exposure to 50 μM BHPF for 24 h (Jiao et al., 2019; Zhang et al., 2021). Thus, our results indicated that H295R cells were sensitive to BHPF exposure. Since endocrine disrupting compounds (EDCs) can interfere with the endocrine system at concentrations that do not cause overt toxicity like inhibition of cell proliferation, the effects of BHPF on steroidogenesis and underlying mechanisms were investigated based on dosages of BHPF that had no remarkable cytotoxicity.

To the best of our knowledge, this is the first report of the adverse effects of BHPF in the basal and dbcAMP-induced treatments on steroidogenesis in H295R cells. The dbcAMP, a derivative of cAMP, was used to mimic the secretion mode of adrenocorticotropic hormone (ACTH) (Feng et al., 2016) and activate the PKA signaling pathway to induce the secretion of progesterone and cortisol, as well as to increase the expression of critical genes involved in the pathway of steroid hormone synthesis in H295R cells (Li et al., 2013). In our study, the significant inhibition of the production of aldosterone, cortisol, testosterone and estradiol observed in H295R cells exposed to BHPF at greater concentrations in both basal and dbcAMP-induced treatments were similar to previous findings that BPA, BPS and BPAF could decrease the production of aldosterone and cortisol in H295R cells treated with dbcAMP (Feng et al., 2016). In addition, BPB was demonstrated to decrease the cortisol level in H295R cells (Rosenmai et al., 2014). Noticeably, the amount of testosterone produced was altered in a non-monotonic profile in H295R cells exposed to BHPF in the basal and dbcAMP-induced treatments. More testosterone was produced at lesser concentrations of BHPF, but less was produced at greater exposures of BHPF. However, BPA, BPS and BPAF were found to decrease testosterone production in a dose-dependent manner in H295R cells (Goldinger et al., 2015; Feng et al., 2016). Additionally, BHPF interfered with estradiol production in a non-monotonic profile similar to that with testosterone production in the basal treatment but different from the findings that BPA, BPF, BPB and BPE stimulated E_2 production (Rosenmai et al., 2014; Goldinger et al., 2015). The non-monotonic responses of BHPF on the production of testosterone and E_2 probably indicated that complicated effects of BHPF on the sex hormone endocrine system, which is associated with reproductive functions. In addition, the effects

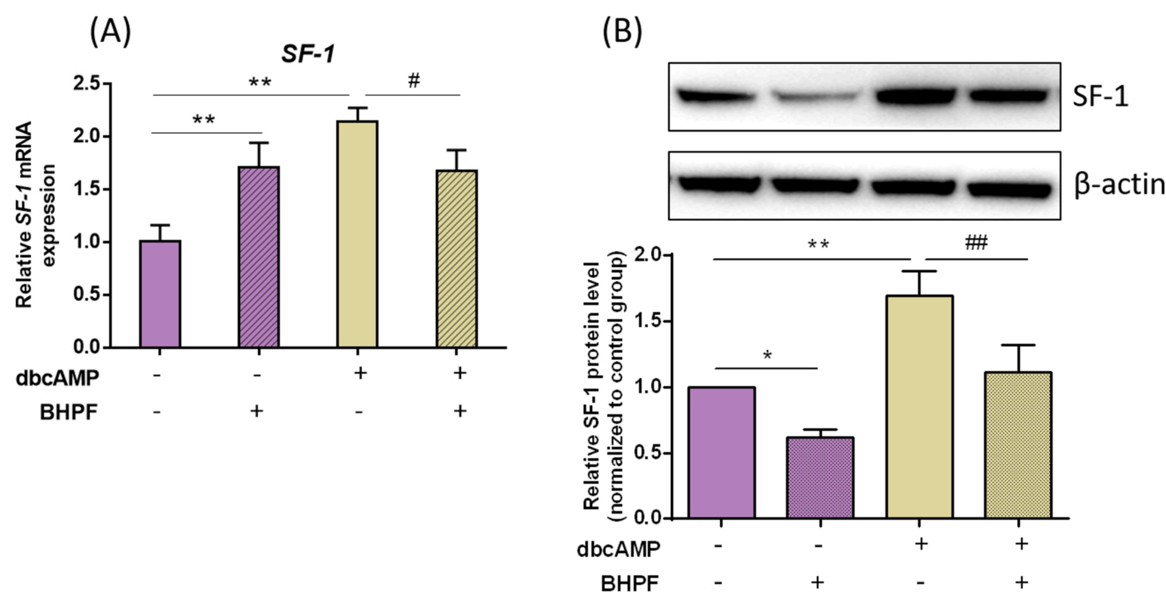

Fig. 6. Effects of BHPF on the gene and protein expressions of SF-1. The H295R cells were exposed to 30 μM BHPF in the basal treatment or 100 μM dbcAMP-induced treatment for 48 h. (A) the relative gene expression of SF-1; (B) the bands of SF-1 expression by western blotting, with *β*-actin used as an internal reference; (C) the alteration of the protein expression level of SF-1. Results are means ± SD of three replicate samples. Compared to the DMSO control, * $p < 0.05$ and * * $p < 0.01$; compared to the dbcAMP-induced control, $^{\#}p < 0.05$ and $^{\#\#}$ $p < 0.01$.

of BHPF on steroidogenesis in H295R cells were partially different from those of BPA and other analogues, suggesting the need for study on endocrine disrupting effects for each BPs.

To elucidate the underlying mechanism of the effects on steroidogenesis of BHPF, transcriptions of 10 critical genes involved in steroidogenesis were analyzed. HMGR and StAR, the essential rate-limiting enzymes, play pivotal roles in cholesterol synthesis and the transfer from the outer mitochondrial membrane to the mitochondrial membrane, respectively (Yan et al., 2018). CYP11A1 and HSD3B2 catalyze cholesterol to produce progesterone, an essential precursor for other steroid hormones synthesis. Progesterone was catalyzed sequentially by CYP21A2, CYP11B1 and CYP11B2 to produce aldosterone (Nogueira and Rainey, 2010). And 17α-hydroxyprogesterone, which is converted from progesterone by CYP17A1, is catalyzed by CYP21A2 and CYP11B1 to produce cortisol (Wang et al., 2015). Hence, CYP11B1 and CYP11B2 are responsible for regulating the final rate-limiting of the synthesis of cortisol and aldosterone, respectively. In our research, 30 μM BHPF could inhibit the aldosterone and cortisol production through the down-regulation of *HMGR*, *StAR*, *CYP11A1*, *HSD3B2*, *CYP11B1* and *CYP11B2* in the basal and dbcAMP-induced treatments. Consistent with the findings of BHPF in our study, BPA, BPF and BPAF can block the synthesis of aldosterone and cortisol via down-regulating the transcription of the rate-limiting enzymes (Feng et al., 2016). However, the reduction in the production of aldosterone and cortisol was observed in 20 μM BHPF dbcAMP-induced group, concurrent with no significant alteration in expressions of StAR, CYP11B1 and CYP11B2. Similarly, exposure to BPS suppressed the production of aldosterone and cortisol with no changes in transcription of their rate-limiting enzymes (Feng et al., 2016). Greater concentrations of BHPF inhibited the production of aldosterone and cortisol, similar to other BPs, but through different mechanisms in the steroidogenic pathway.

CYP17A1 catalyzes the conversion of progesterone and 17α-hydroxyprogesterone to androstenedione, which is the precursor substance from which testosterone and E_2 are produced. 17βHSD and CYP19A1 catalyze the rate-limiting reactions for the synthesis of testosterone and E_2, respectively (Oskarsson et al., 2006). In the basal treatment, BHPF might inhibit testosterone production by down-regulation of *CYP17A1* and *17βHSD*. In contrast, due to no significant alteration in expression of *17βHSD*, the down-regulation of *CYP17A1* mainly reduced testosterone production in H295R cells exposed to BHPF in the dbcAMP-induced treatment. Similarly, exposure to BPA, BPF, BPS or BPAF decreased testosterone production by down-regulation of *CYP17A1* rather than *17βHSD,* which was the case in dbcAMP-induced H295R cells (Feng et al., 2016). Since expressions of *CYP17A1* and *17βHSD* were not significantly altered after H295R cells were exposed to 10 μM BHPF in the basal treatment, a decrease in testosterone production occurred might be due to down-regulation of expression of other genes in the upstream steroidogenic pathway or changes in the translation of mRNA or post-modification of proteins. Results of this study indicated that the disrupting effect of BHPF on steroidogenesis for H295R cells was different in the presence and absence of dbcAMP and might be a function of exposure concentrations, as well as other confounders. Noticeably, lesser concentrations of BHPF might stimulate testosterone production in the basal and dbcAMP-induced cells through up-regulation of *17βHSD*. Also, down-regulation of expression of *CYP19A1* might result in lesser conversion from testosterone to E_2, thereby promoting testosterone secretion. The non-monotonic effect of BHPF on synthesis of testosterone was different from what has been observed for other BPs that only caused suppression (Zhang et al., 2011; Rosenmai et al., 2014; Goldinger et al., 2015). It suggested that more concerns about effects of BHPF on the androgen endocrine system and male reproduction are necessary, especially exposures to BHPF at low concentrations.

The down-regulated transcription of *CYP19A1* was observed in a dose-dependent manner, whereas only BHPF at a concentration of 30 μM exhibited inhibition of E_2 production. Assuming that the enzyme directly participates in regulation of steroidogenesis and down-regulated transcription levels of genes might be accompanied by unchanged or even increased amounts of protein. Thus down-regulation of *CYP19A1* was not bound to decrease E_2 production, especially accompanying no remarkable alteration in upstream genes of the steroidogenic pathway. Exposure to BPF stimulated E_2 production without changes in transcription of *CYP19A1*. Also, no significant alteration in expression of *CYP19A1* was observed after exposure to BPA, BPS or BPAF (Feng et al., 2016). Compared to that finding, BHPF exerted a more sensitive effect on transcription of *CYP19A1* than did other BPs. Thus, this might be used as a biomarker specific to the endocrine-disrupting effects of BHPF. Furthermore, down-regulation of *CYP19A1* further demonstrated the

anti-estrogen activity of BHPF, which has been reported before (Zhang et al., 2017). Whether CYP19A1 could be a biomarker indicative of endocrine-disrupting effects deserves further investigation.

The cAMP/PKA signaling pathway plays an essential role in steroidogenesis. Activation of AC increases intracellular levels of the secondary messenger cAMP, which ultimately activates the PKA signaling pathway. Steroidogenesis is mediated by the intracellular cAMP content, which has been reported to stimulate production of steroid hormones (Yan et al., 2018; Jin et al., 2020). When activated PKA promotes phosphorylation of downstream target proteins and then recruits some co-activators, such as the cAMP response element-binding protein (CREB)-regulated transcription coactivators (CRTC) and SF-1 forming a complex to mediate steroidogenic acute regulatory protein (StAR) and expressions of cytochrome P450 1B1 (CYP1B1) expressions (Zheng and

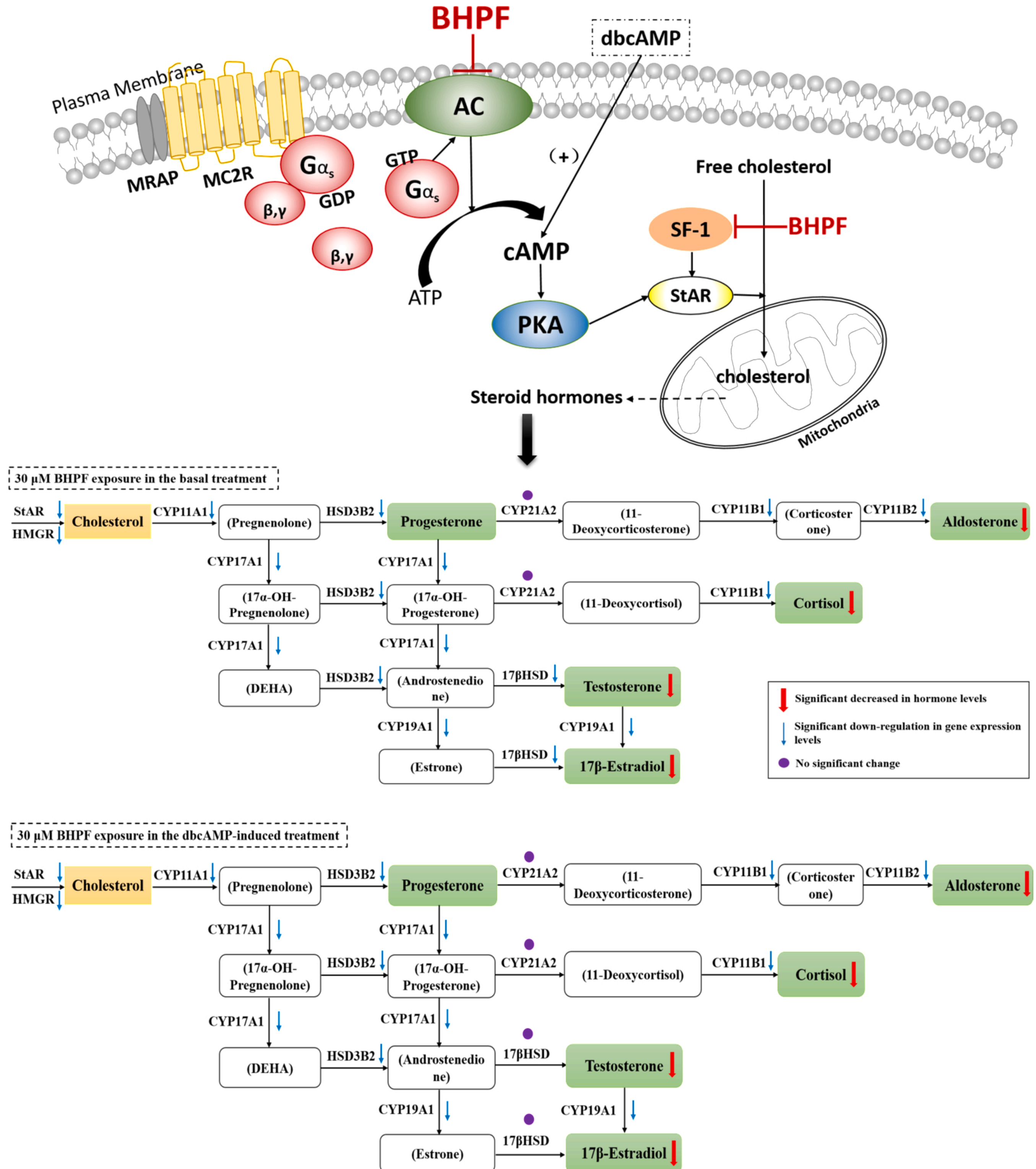

Fig. 7. Schematic illustration of the mechanisms by which BHPF attenuates steroidogenesis through suppression of the AC/cAMP/PKA pathway and down-regulation of SF-1 in H295R cells.

Jefcoate, 2005; Smith et al., 2020). In the present study, BHPF significantly reduced concentrations of intracellular cAMP via attenuating the AC activity induced by FSK, an activator of AC activity. The decreased cAMP content led to inhibition of PKA activation in both BHPF dbcAMP-induced and FSK-induced cells. However, only significant inhibition of PKA activity was observed in the BHPF basal treatment, indicating that distinct profile of the AC/cAMP/PKA signaling pathway was involved between the basal and dbcAMP-induced treatments. Noticeably, PKA might play a pivotal role in BHPF-induced suppression of steroidogenesis. Alteration of PKA activity contributed to BPA-induced dysfunction of spermatozoa in adult rats (Wan et al., 2016) or F1 adult mice (Rahman et al., 2017). Additionally, further study on its downstream signal molecules, such as CREB, CRTC, and SF-1, is essential due to their important regulation in the transcription of steroidogenic genes. A recent report demonstrated that BPA has sex-specific effects on learning and memory, which is associated with down-regulation of PKC/ERK/CREB signaling pathway (Wu et al., 2020).

Previous reports have demonstrated that SF-1, a key steroidogenic transcription factor, plays a pivotal role in steroidogenesis by regulating the transcription of steroidogenic genes, including StAR, CYP11A1, CYP1B1, and CYP19A1 (Meinsohn et al., 2019; Liu et al., 2020). Although it has been found that BPA could not alter adrenal expression of SF-1 in adult mouse offspring (Medwid et al., 2016), our results showed that decreased expression of SF-1 protein after BHPF exposure was directly involved in down-regulation of critical steroidogenic genes in both the basal and dbcAMP-induced treatments. Interestingly, BHPF could stimulate the transcription of *SF-1* while inhibiting the protein expression of SF-1 in the basal treatment. It suggested that BHPF might be inclined to inhibit translation of SF-1 and even post-translation modification, ultimately leading to reduced protein expression of SF-1. The inconsistency of changes in SF-1 gene and protein expression after BHPF basal exposure might be associated with post-transcriptional regulation, indicating that more complicated mechanisms may be involved in the effects of BHPF on steroidogenesis.

5. Conclusions

In conclusion, our findings firstly demonstrated that BHPF could alter steroidogenesis in H295R cells at concentrations that did not alter proliferation. Relative in the basal and dbcAMP-induced cells, steroidogenesis was decreased due to down-regulation of steroidogenic genes in cells exposed to greater concentrations of BHPF. Blockage of the AC/cAMP/PKA pathway and down-regulation of SF-1 were involved in BHPF-suppressed steroidogenic gene expressions and steroidogenesis (Fig. 7). Further in vitro and in vivo studies are needed to elucidate the mechanisms of the endocrine disruption effects and assess the potential health risks of BHPF, especially BHPF exposure at low concentrations.

CRediT authorship contribution statement

Yuan Huang: Conceptualization, Methodology, Formal analysis, Data curation, Visualization, Investigation, Writing – original draft, Writing – review & editing; **Wei Zhang:** Writing – review & editing, Conceptualization, Validation; **Na Cui:** Data curation, Validation; **Zhiming Xiao:** Conceptualization, Resources; **Wenyu Zhao**: Investigation; **Ruiguo Wang**: Resources; **John P. Giesy:** Writing – review & editing; **Xiaoou Su:** Project administration & supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113982.

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Supplementary materials

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Fluorene-9-bisphenol regulates steroidogenic hormone synthesis in H295R cells through the AC/cAMP/PKA signaling pathway

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Yuan Huang [a], Wei Zhang [a, *], Na Cui [a], Zhiming Xiao [a], Wenyu Zhao [a], Ruiguo Wang [a], John P. Giesy [b, c, d], Xiaoou Su [a, *]

[a] *Institute of Quality Standards and Testing Technology for Agro-Products, Chinese Academy of Agricultural Sciences, Beijing 10081, China*

[b] *Department of Veterinary Biomedical Sciences, and Toxicology Center, University of Saskatchewan, 52 Campus Dr, Saskatoon, SK S7N 5B4, Canada*

[c] *Department of Integrative Biology and Center for Integrative Toxicology, Michigan State University, 784 Wilson Rd, East Lansing, MI 48824, USA*

[d] *Department of Environmental Science, Baylor University, 97266 One Bear Place, Waco, TX 76798, USA*

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*Corresponding author:

E-mail: suxiaoou@caas.cn (X. Su), zhangwei03@caas.cn (W. Zhang)

Tel: +86-010-82106581

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29 **Table S1.** Sequences of primers used for real-time RT-PCR amplification in this study.

Target gene	GenBank accession No	Primer sequences	Product length (bp)
HMGR	NM_000859	FW: TGCTTGCCGAGCCTAATGAAAG	152
		RW: AGAGCGTTCGTGGGTCCATC	
StAR	NM_000349	FW: TACGTGGCTACTCAGCATCGAC	139
		RW:TCAACACCTGGCTTCAGAGGCA	
CYP11A1	NM_000781.3	FW: GAGATGGCACGCAACCTGAAG	137
		RW: CTTAGTGTCTCCTTGATGCTGGC	
CYP11B1	NM_000497.3	FW: CTTCCAGTACGGCGACAA	112
		RW: CGACAGTTCCGCATTCAA	
CYP11B2	NM_000498.3	FW: TCCAGGTGTGTTCAGTAGTTCC	146
		RW: GAAGCCATCTCTGAGGTCTGTG	
CYP17A1	NM_000102.4	FW: AGCCGCACACCAACTATCAG	134
		RW: TCACCGATGCTGGAGTCAAC	
CYP19A1	NM_000103.4	FW: AGGTGCTATTGGTCATCTGCTC	128
		RW: TGGTGGAATCGGGTCTTTATGG	
CYP21A2	NM_000500.9	FW: CGTGGTGCTGACCCGACTG	108
		RW: GGCTGCATCTTGAGGATGACAC	
17βHSD	NM_000413.4	FW: CTCCCTCTGACCAGCAACC	136
		RW: TGTGTCTCCCACGCAATCTC	
HSD3B2	NM_000198.4	FW: TGCCAGTCTTCATCTACACCAG	95
		RW: TTCCAGAGGCTCTTCTTCGTG	
SF-1	U76388.1	FW: GGAGTTTGTCTGCCTCAAGTTCA	80
		RW: CGTCTTTCACCAGGATGTGGTT	
β-actin	NM_001101	FW: CACTCTTCCAGCCTTCCTTCC	100
		RW: AGGTCTTTGCGGATGTCCAC	

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