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Original Article

Impact of low concentrations of phthalates on the effects of 17β-estradiol in MCF-7 breast cancer cells

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ABSTRACT

Objective: To explore whether lower concentrations of phthalates interfere with the effects of 17β-estradiol on the growth of MCF-7 breast cancer cells.
Materials and Methods: MCF-7 cells were treated with 17β-estradiol (E_2), phthalates, including butyl benzyl phthalate (BBP), di(*n*-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP), or with both E_2 and phthalates, all at 10nM. After incubation for 48 hours, the cells were harvested and extracted for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The proteins involving proliferative and apoptotic pathway were then evaluated using Western blot analysis.
Results: In MCF-7 cell cultures, the MTT assay revealed a significant increase in cell viability with E_2 and these three phthalates, and significantly more cell proliferation with the combination of E_2 and phthalates. Proliferating cell nuclear antigen, as well as phosphatidylinositide 3-kinase (PI3K) and p-Akt, were all substantially increased in cultures with E_2, phthalates, and the two combined. An additive effect of phthalates on the obvious increase of Bcl-2 and ER α expression was also noted in the presence of E_2.
Conclusion: The present study demonstrates that even at a very low concentration, BBP, DBP, and DEHP were not only still capable of displaying estrogenic activity, but also of inducing an additive proliferative effect through the PI3K/Akt signaling pathway and preventing apoptosis in the presence of E_2. Therefore, the effects of current reference doses for phthalates defined by the government, especially for premenopausal women, should be further considered.

Introduction

Phthalates are widely used in our general life and are generally recognized as an endocrine disrupter because of their estrogenic and antiandrogenic activities. Therefore, whether or not phthalates play a role in steroid hormone-dependent cancers, such as breast cancer, has been strongly considered. Although the association between phthalates exposure and the risk of breast cancer is still controversial, epidemiological evidence suggests that the risk of breast cancer increases following exposure to diethyl phthalate in the environment [1]. A Canadian case–control study revealed that women working in the automotive and food canning industries have nearly a five-fold increase in risk for premenopausal breast cancer, likely because of their exposure to phthalates [2]. Thus, exposure to phthalates becomes an important issue, especially for premenopausal women, for whom exposure might induce higher risk for breast cancer.

Although there is increasing concern that phthalates may interfere with endogenous estrogens, there is still discrepancy on the effects of health risks induced by the combination of phthalates and endogenous estrogen. It has been considered that xenoestrogens—with their weaker estrogenic activity and low levels in human tissue—may not induce any harm, because they cannot impact the strong estrogenic effects and higher levels of endogenous steroid estrogen [3]. By contrast, Rajapakse et al [4] showed that weak xenoestrogens can significantly modulate the effects of 17β-estradiol, even when each compound was present below the concentration at which no effect was observed. One study demonstrated that the combination of estradiol and the weak

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xenoestrogens bisphenol A and *o,p′*-DDT induced synergistic effects [5]. Another study revealed that polybrominated diphenyl ethers induced an additive antiapoptotic and proliferative effects in the presence of 17β-estradiol [6]. It has also been reported that the interaction of phytoestrogens with 17β-estradiol is dose dependent, i.e., low concentrations of genistein and coumestrol significantly enhanced 17β-estradiol-induced DNA synthesis in MCF-7 cells, whereas high concentrations caused inhibition [7]. Thus, whether synthetic or natural xenoestrogens may modulate the effects of endogenous 17β-estradiol should be further evaluated.

Phthalates, including butyl benzyl phthalate (BBP), di(*n*-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP), are synthetic xenoestrogens that are commonly used in consumer products and are capable of binding to estrogen receptors (ERs) [8–10]. Female sex hormones are linked to the etiopathogenesis of breast cancer in which estrogen regulates cellular responses by binding to ERs, regulating transcription of target genes in the nucleus, and activating a signaling pathway in the cytoplasm. Therefore, in the presence of endogenous estrogen, the potential effects that may be induced or modulated as a result of these phthalates and their ER binding capabilities should be a cause for concern.

The incidence of breast cancer in Taiwan continues to increase, with an average age that is 10 years younger than their European and American counterparts. European women tend to develop breast cancer between the ages of 55 years and 65 years, whereas Taiwanese women tend to develop the disease when they are between 45 years and 55 years old. It has been suspected that the younger onset of breast cancer in Taiwan could be potentially related to general lifestyle practices such as using plastic bags as containers for hot food for the sake of convenience.

Therefore, in order to investigate the effects of phthalates in relation to potent steroid estrogen, we studied, in the presence of 17β-estradiol, both the effects and signaling pathway induced by BBP, DBP, and DEHP at lower concentrations on the cell growth of MCF-7 human breast cancer cells.

Materials and methods

Ethics

This study was approved by the Ethical Medicine Committee of Chang Gung Memorial Hospital, Keelung, Taiwan and supported by the Clinical Monitoring Research Program of Chang Gung Memorial Hospital.

Study design

MCF-7 cells were treated with a control medium, 10^{-8}M 17β-estradiol (E_2) (physiological doses: from 10^{-9}M to 10^{-7}M), 10nM phthalates (BBP, DBP, and DEHP), and a combination of one of these phthalates plus E_2 for 48 hours, and were then extracted for a cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], and a cell death assay [dUTP nick end labeling (TUNEL) assay]. Then, we further investigated their proliferative and apoptotic features, in addition to their effects on ER α, after the cells were incubated with these agents. The phosphatidylinositide 3-kinases (PI3K)/Akt pathway mediates the effects of a variety of extracellular signals in a number of cellular processes, including cell growth, proliferation, and survival, which in turn is involved in cancer progression. Thus, we examined the alteration of integrants of this signaling pathway in breast cells, including p-PI3K, p-Akt, and proliferating cell nuclear antigen (PCNA). Apoptosis is triggered through the intrinsic (mitochondrial) and extrinsic (Fas/Fas-L) pathways to activate caspase proteases [11]. Bcl-2 prevents the activation of caspase-3 in extracts lacking mitochondria, and consequently blocks a pathway leading to the activation of a caspase cascade. Thus, we observed both the zymogen and cleaved forms of caspases, including Bcl-2, Fas-L, tBid, and caspase-3. The detailed experiment methods are described as follows. We decided to use a 10nM concentration of phthalate because of its effects on cell viability as observed in our previous studies using MCF-7 breast cancer cells [12].

Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI-1640 phenol red-free medium supplemented with 10% charcoal/dextran-treated fetal bovine serum, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin, 1mM nonessential amino acids, and 1mM sodium pyruvate in humidified air (5% CO_2) at 37°C. MCF-7 cells were cultured in a serum-free medium for 12 hours and then treated with 0.1% EtOH as a control medium or with BBP (10nM; Accustandard, New Haven, CT, USA), DBP (10nM; Accustandard), DEHP (10nM; Sigma, St Louis, MO, USA), and E_2 (10nM; Sigma) either alone or in conjunction (10nM E_2 with 10nM BBP, DBP, or DEHP). After further incubation for 48 hours, the cells were harvested and extracted for subsequent analysis.

Cell proliferation assay (MTT assay)

To determine the cell proliferation and viability, MCF-7 cells were cultured in 24-well plates (1×10^5 cells/well). After treatment with the aforementioned compounds for 48 hours, respectively, cell viability was determined using an MTT assay (Sigma). The absorbance of blue formazan crystals was measured at 570 nm using a spectrophotometer (Molecular Device Spectramax M3; Molecular Devices, Sunnyvale, CA, USA). The quantity of the formazan product was directly proportional to the number of viable cells in the culture medium.

Measurement of cell death (TUNEL assay)

Cell death (apoptosis) was measured by terminal deoxynucleotidyl transferase-mediated TUNEL assay. MCF-7 cells grown on 6-mm plate were washed twice with phosphate-buffered saline (PBS) and fixed for 30 minutes in 4% buffered paraformaldehyde. The cells were then incubated with 0.1% Triton X-100 in 0.1% sodium citrate solution for 8 minutes, washed in PBS, and incubated with terminal deoxynucleotidyl transferase for 90 minutes and fluorescein isothiocyanate-dUTP for 30 minutes at 37°C using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope at this state by an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green).

Protein extraction and Western blot analysis

Cultured MCF-7 cells were scraped and washed once with PBS. The cell suspension was then centrifuged. The cell pellets were lysed for 30 minutes in a lysis buffer (50mM Tris, pH 7.5, 0.5M NaCl, 1.0mM EDTA, pH 7.5, 10% glycerol, 1mM basal medium Eagle, 1% Igepal-630, and proteinase inhibitor cocktail tablet; Roche Applied Science) and then centrifuged at 12,000*g* for 10 minutes. The supernatants were removed and placed in new Eppendorf tubes for Western blot analysis. Proteins from MCF-7 cells were separated in 12% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes.

Nonspecific protein binding was blocked in blocking buffer at room temperature for 1 hour (5% milk, 20mM Tris–HCl, pH 7.6, 150mM NaCl, and 0.1% Tween 20). The membranes were blotted with specific caspase-3 (Chemicon, Temecula, CA, USA), truncated Bid (tBid; Santa Cruz Biotechology, Santa Cruz, CA, USA), Fas ligand (Fas-L; Chemicon), Bcl-2 (BD Biosciences, San Jose, CA, USA), phosphorylation-phosphatidylinositide 3-kinases (p-PI3K; Upstate Biotechnology, Lake Placid, NY, USA), phosphorylation-Akt (p-Akt; Chemicon), PCNA (Chemicon), phosphorylated ER α (Upstate Biotechnology), and β-actin (Upstate Biotechnology) antibodies and incubated in 4°C blocking buffer overnight. Densitometric analysis of immunoblots was performed using the Bio-Rad molecular imager versadoc MP 4000 system (Bio-Rad, Hercules, CA, USA). The experiments were performed in triplicate.

Statistical analysis

Data for cell proliferation were expressed as percentage, which were arbitrarily assigned 100%. All data were measured versus controls and performed in triplicate. The statistical significance of differences was calculated using Student *t* test for paired data with the level of significance set at $p \leq 0.05$. All of the data were analyzed using Excel statistical software (Microsoft Corporation, Redmond, WA, USA).

Results

Effects of phthalates and 17β-estradiol on cell viability of MCF-7 cells

In Figure 1, BBP, DBP, DEHP, and E_2 alone significantly increased cell proliferation. An additive increase in cell proliferation was noted after combined treatment with E_2 and one of these three phthalates as compared with the control or phthalates alone.

Phthalates and 17β-estradiol induce proliferation of MCF-7 cells through PI3K/Akt signaling pathway

In Figures 2A–2C, p-PI3K, p-Akt, and PCNA were all significantly increased in cultures with E_2 and phthalates (BBP, DBP, and DEHP) alone, and the effects were even more pronounced after combined treatment with phthalates and E_2.

Apoptosis effects of phthalates and 17β-estradiol in MCF-7 cells

As shown in Figure 3A, in MCF-7 cells, Bcl-2 significantly increased in cultures with BBP alone, DBP alone, E_2 alone, and a combination of E_2 and one of these three phthalates as compared to the control. However, only E_2 alone suppressed the expression of Fas-L (Figure 3B) and tBid (Figure 3C). Caspase-3 significantly decreased in cultures with E_2 alone, but there were no substantial changes in caspase-3 expression between the control and phthalate- or phthalate plus E_2-treated MCF-7 cells (Figure 3D). The effects on caspase-3 activity were further confirmed by TUNEL assay, in which apoptotic cells were significantly decreased only in the cultures using E_2 alone (Figure 3E).

Effects of phthalates and 17β-estradiol on ER α expression in MCF-7 cells

As shown in Figure 4, the expression of ER α phosphorylation was notably increased in MCF-7 cells treated with E_2, BBP, DBP, and DEHP alone. An additive increase in ER α expression cell proliferation was noted after combined treatment with E_2 and one of these three phthalates as compared to the control.

Discussion

The present study demonstrates that even low concentrations of BBP, DBP, and DEHP are able to significantly modulate the effects of

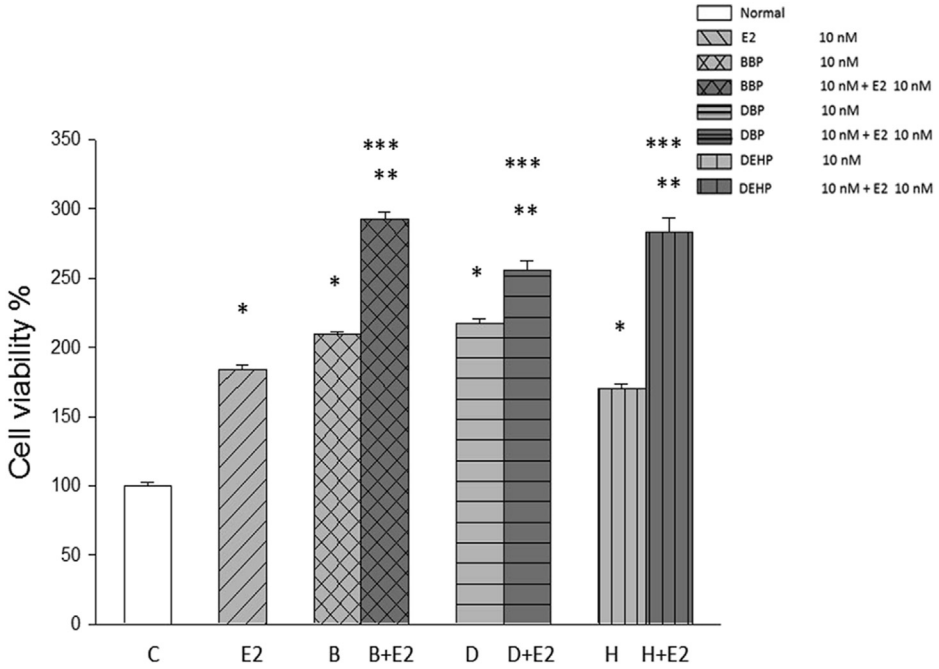


Figure 1. Effects of 17β-estradiol (E_2), butyl benzyl phthalate (BBP), di(*n*-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP) alone or a combination of E_2 and one of these three phthalates on cell proliferation of MCF-7 human breast cancer cells. Cells were exposed to control (0.1% EtOH in medium), E_2 (10nM), BBP (10nM), DBP (10nM), DEHP (10nM), or a mixture of E_2 and one of these three phthalates at the concentrations of 10nM and incubated for 48 hours. All data are reported as the percentage change in comparison with the vehicle only group, which were arbitrarily assigned 100% viability. Data are presented as mean ± standard deviation. $^{*}p < 0.05$ versus control, $^{**}p < 0.001$ versus control, $^{***}p < 0.05$ versus phthalates. B = BBP; C = control; D = DBP; H = DEHP.

potent 17β-estradiol on breast cancer cells *in vitro*. Although the combination of phthalates and E_2 do not have a comparable effect as E_2 on apoptosis of breast cancer cells, they do inhibit the intrinsic pathways of apoptosis. The additive proliferative effects of BBP, DBP, and DEHP on breast cancer cells treated with E_2 may be related to the joint effect of their affinity to ER α.

Phthalates, including BBP, DBP, and DEHP, are used as plasticizers, which are also widely used in food wraps and cosmetic

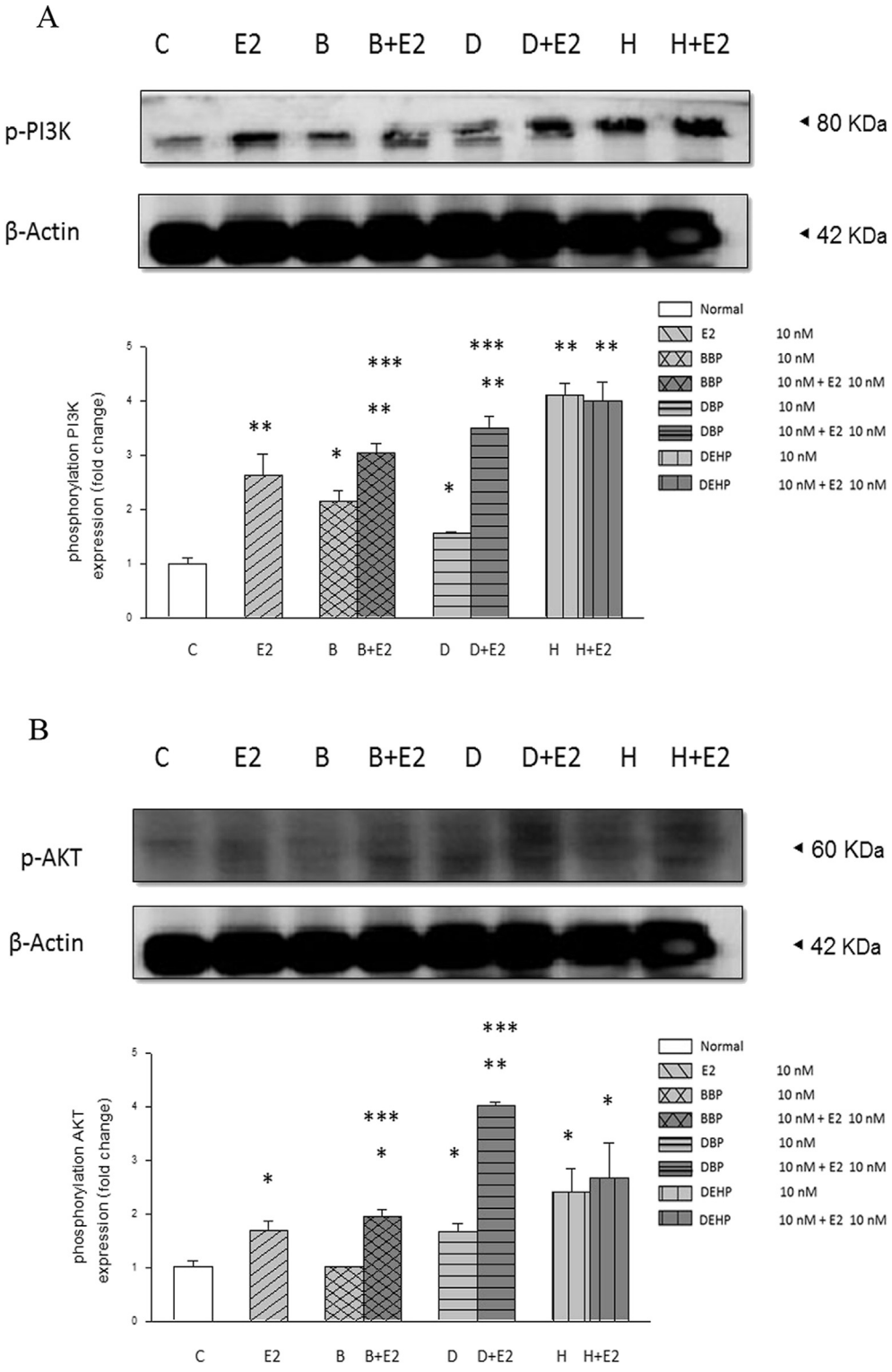


Figure 2. Effects of 17β-estradiol (E_2), butyl benzyl phthalate (BBP), di(*n*-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP) alone or a combination of E_2 and one of these three phthalates on (A) phosphorylation phosphatidylinositide 3-kinases (p-PI3K), (B) phosphorylation-Akt (p-Akt), and (C) proliferating cell nuclear antigen (PCNA) of MCF-7 human breast cancer cells. Cells were exposed to control (0.1% EtOH in medium), E_2, BBP, DBP, DEHP, or a mixture of E_2 and one of these three phthalates all at the concentrations of 10nM and incubated for 48 hours. p-PI3K and p-Akt were measured using immunoblotting assay. $^{*}p < 0.05$ versus control; $^{**}p < 0.001$ versus control; $^{***}p < 0.05$ versus phthalates. B = BBP; C = control; D = DBP; H = DEHP.

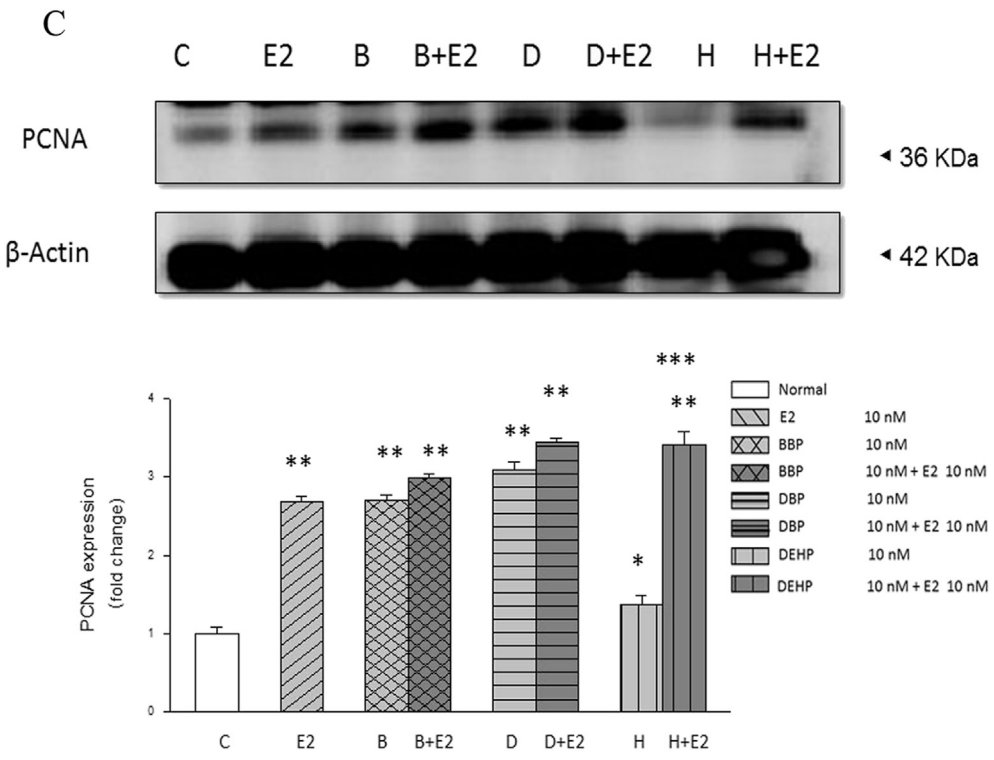


Figure 2. (*continued*).

formulations. Our previous study investigated the effects of BBP, DBP, and DEHP at various concentrations on breast cancer cells. We found that even at a low concentration (10nM), these phthalates not only induced a proliferative effect, but also demonstrated estrogenic effects as well [12]. Thus, the risk of breast cancer associated with the exposure to phthalates should be of concern. Evidence from the U.S. National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) database suggests that the incidence of advanced breast cancer in young women is increasing [13]. Thus, although phthalates have a low potency of estrogenic activity as compared with endogenous steroid estrogens and have low levels in human tissues, it should be further considered whether the interaction of phthalates with endogenous estrogens may induce negligible or synergistic effects. In the present study, we examined the cell viability of mixtures of E_2 and BBP, DBP, or DEHP on MCF-7 human breast cancer cells, which revealed additive proliferation effects. Although several studies have also revealed that these phthalates increases the proliferation ability of MCF-7 breast cancer at various concentrations [8,14,15], to our knowledge, this is the first report to demonstrate that cotreatment of E_2 and these phthalates additionally increases E_2-stimulated breast cancer cells proliferation *in vitro*. In order to realize the actual effects of combined E_2 and phthalates, we further investigated both their proliferative and apoptotic features in this study.

Akt pathways play critical roles in mammalian cell survival signaling and have been believed to be an attractive target for cancer prevention or treatment [16]. In the present study, the phosphorylated PI3K, Akt, and PCNA proteins were significantly increased after E_2, phthalates (BBP, DBP, and DEHP) alone, or a combination of phthalates and E_2, which indicated their capabilities to induce cellular proliferation. It has been reported that estrogens may induce Akt activity in ER-negative breast cancer cells [17]. Hsieh et al [18] also observed Akt activation by BBP and DBP at a higher concentration (10^{-6}M) in human breast epithelial stem cells. The upregulation of Akt signaling pathways may be one of the molecular mechanisms that induce the additive effects noted after combined treatment with E_2 and one of these phthalates.

In mammalian cells, two major signaling pathways leading to cell death by apoptosis have been identified: the intrinsic pathway (or mitochondrial pathway) and the extrinsic pathway (or the death receptor, Fas/Fas-L, pathway). In the present study, E_2 prevented apoptosis by upregulation of Bcl-2 expression and downregulation of Fas-L and tBid expression, which resulted in the significant decrease of caspase-3 expression. Cotreatment of E_2 and one of these three phthalates induced additive regulation of Bcl-2 expression and nonsignificant changes on Fas-L and tBid expression, which resulted in nonsignificant effects on caspase-3 activity. These conditions were further confirmed by TUNEL assay. The results further support the assertion that the apoptosis inhibited by E_2 was associated with both the intrinsic and extrinsic apoptosis pathways. Gompel et al [19] also found results on E_2 regulation of apoptosis in breast cancer cell lines similar to our study, in which E_2 alone did not inhibit cell death but rather, increased the antiapoptotic proteins, bcl-2 and bclx(L). Kim et al [20] also revealed that BBP (100M), DBP (10M), and DEHP (10M) not only significantly increased cell proliferation, but also inhibited tamoxifen-induced apoptosis by an increase in intracellular Bcl-2/Bax ratio in MCF-7 human breast cancer cells. Although individual phthalate had no effects on the extrinsic apoptosis pathways in the present study, it may be related to the very low concentrations used. It is well recognized that the effects of steroid hormone receptor agonist/antagonist not only are determined by ligand–receptor binding, but also depend on interaction with specific effector molecules [21]. Thus, it should be also considered and further evaluated how the convergence of ligand-dependent and ligand-independent mechanism may impact the joint effects of E_2 and these phthalates on the apoptosis pathway.

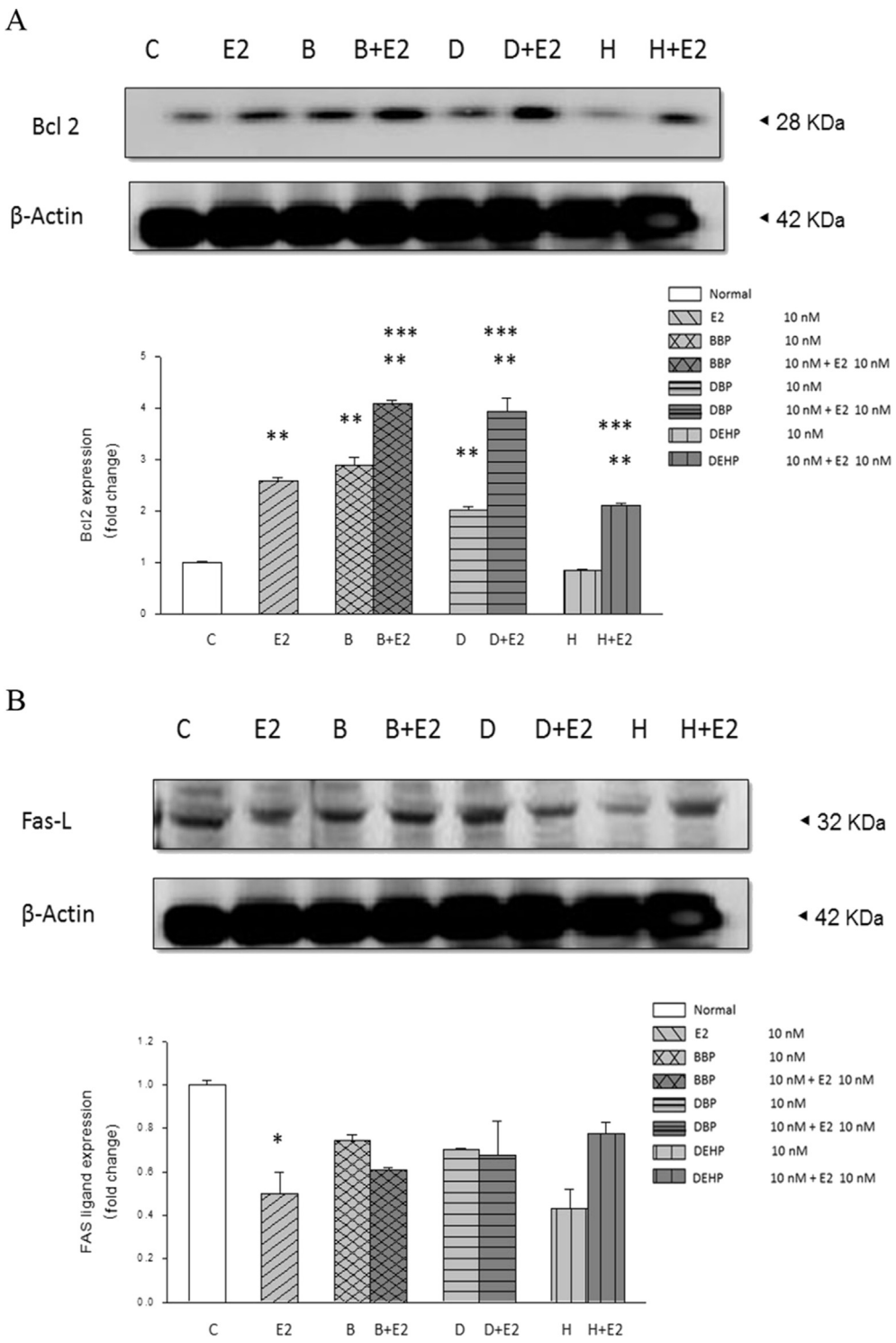


Figure 3. Effects of 17β-estradiol (E_2), butyl benzyl phthalate (BBP), di(*n*-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP) alone or a combination of E_2 and one of these three phthalates on apoptosis (A) Bcl-2, (B) Fas ligand (Fas-L), (C) truncated Bid (tBid), and (D) caspase-3, as well as of MCF-7 human breast cancer cells. Cells were exposed to control (0.1% EtOH in medium), E_2, BBP, DBP, DEHP, or a mixture of E_2 and one of these three phthalates all at the concentrations of 10nM and incubated for 48 hours. (A) Bcl-2, (B) Fas-L, (C) tBid, and (D) caspase-3 were measured using immunoblotting assay. (E) dUTP nick end labeling (TUNEL) assay was uses to detect apoptotic cells. $^{*}p < 0.05$ versus control; $^{**}p < 0.001$ versus control; $^{***}p < 0.05$ versus phthalates. B = BBP; C = control; D = DBP; H = DEHP.

Our previous report has demonstrated that a substantial increase of ER α expression was noted in MCF-7 cells treated with these phthalates or E_2, whereas the ER β expression was significantly reduced in cultures with E_2, but not in cultures with BBP, DBP, and DEHP at a concentration of 10nM [12]. Thus, the present study only evaluated the ER α in MCF-7 breast cancer cells treated with these phthalates and/or E_2, in which an additional increase of ER α expression was noted by cotreatment of E_2 and one of these phthalates. Okubo et al [22] also demonstrated that the stimulation of proliferation of MCF-7 cells by BBP and DEHP at $>10^{-3}$M can be

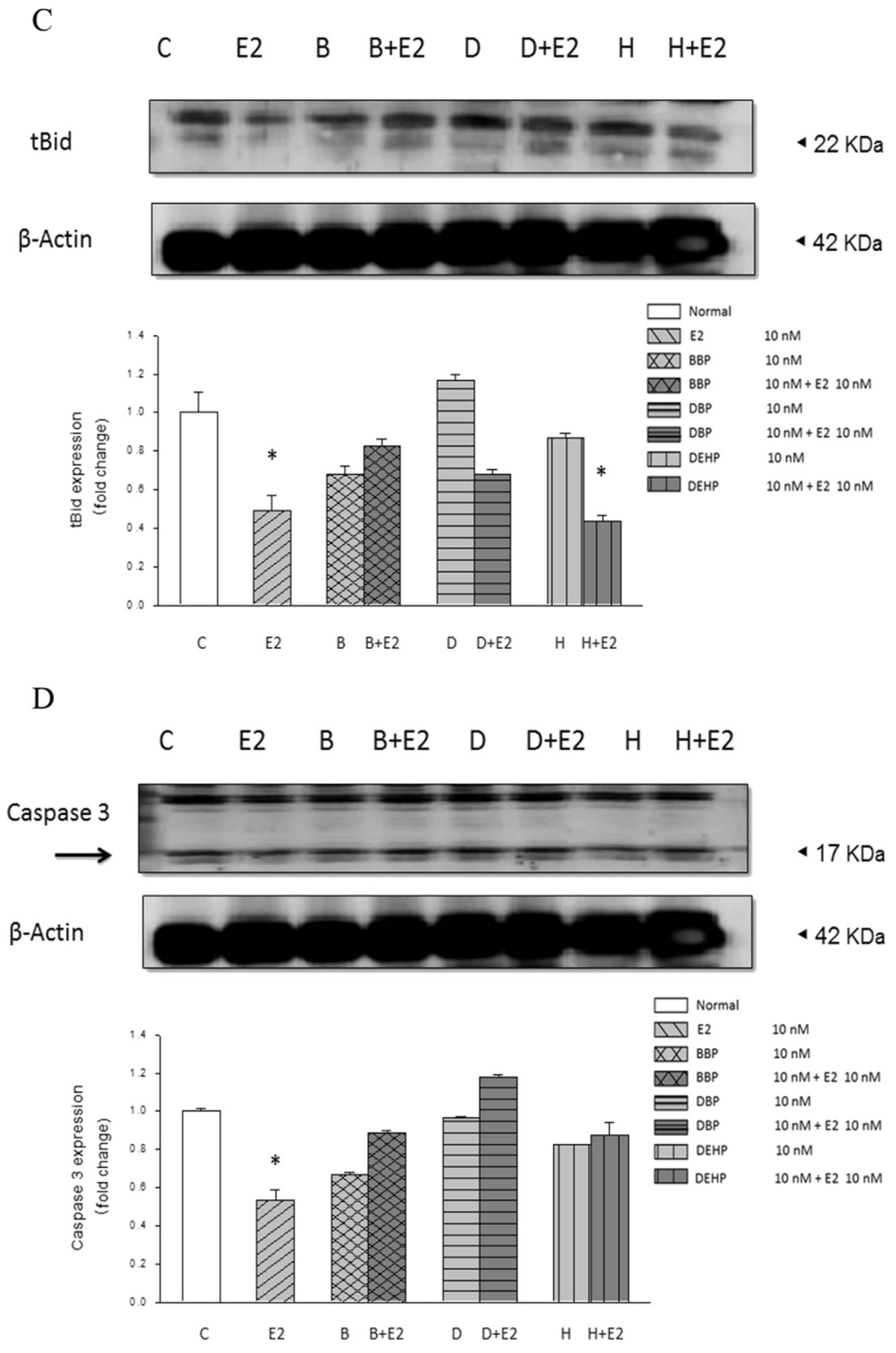
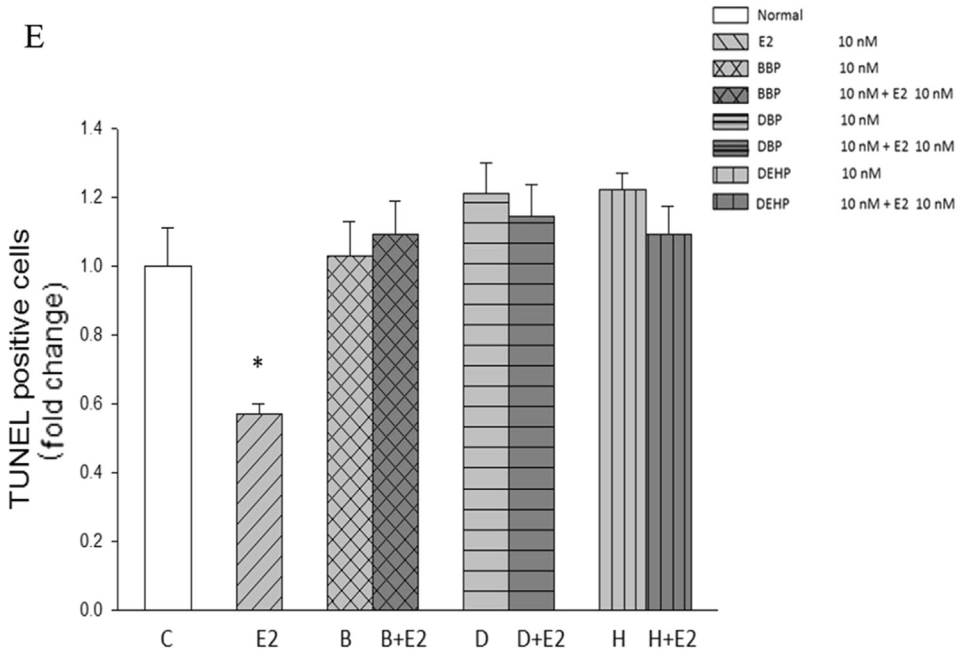


Figure 3. (*continued*).

completely suppressed by the ER antagonist, ICI182780. In the present study, the increased affinity to ER α may be associated with the additively proliferative effects of combined treatment with these phthalates and E_2 in MCF-7 breast cancer cells *in vitro*.

Taken together, the present study demonstrates that even at very low concentrations, BBP, DBP, and DEHP not only induce estrogenic activity and breast cancer cell proliferation, but are also capable of significantly modulating the effects of the potent E_2. BBP, DBP, or DEHP combined with E_2 causes additive effects on estrogenic activity, as well as proliferative effects through the PI3K/Akt signaling pathway. According to the Scientific Committee on Health and Environmental Risks, the recommended tolerable daily intake is as follows: DEHP, 0.05 mg/kg body weight per day; BBP, 0.5 mg/kg body weight per day; DBP, 0.01 mg/kg body weight per day.



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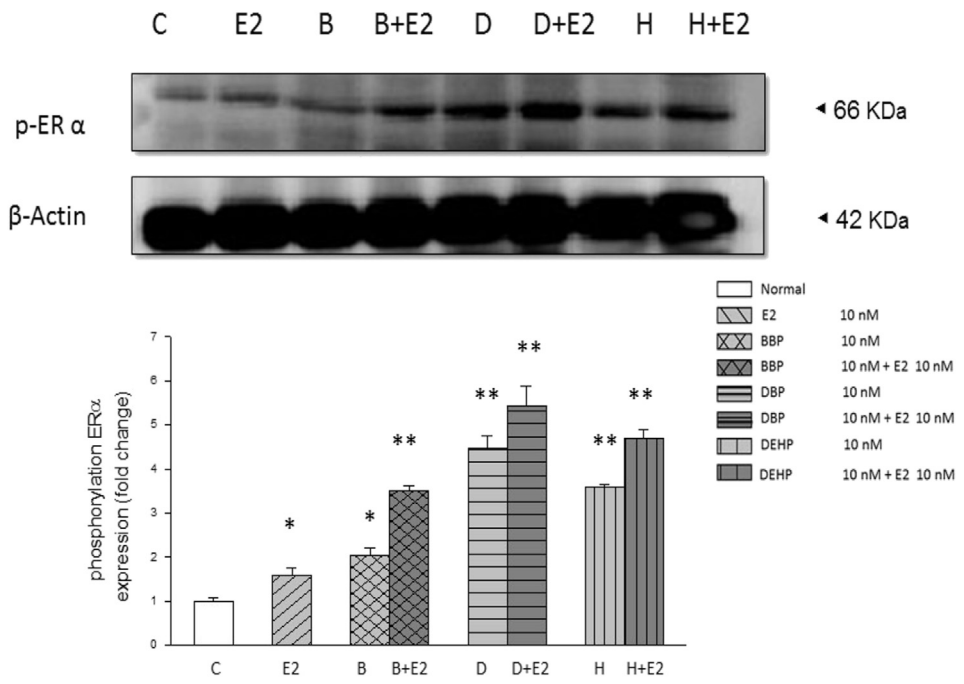

Figure 4. Effects of 17β-estradiol (E_2), butyl benzyl phthalate (BBP), di(*n*-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP) alone or a combination of E_2 and one of these three phthalates on estrogen receptor (ER) α expression of MCF-7 human breast cancer cells. Cells were exposed to control (0.1% EtOH in medium), E_2, BBP, DBP, DEHP, or a mixture of E_2 and one of these three phthalates all at the concentrations of 10nM and incubated for 48 hours. The expression of phosphorylation-ER α (p-ERα) was measured using immunoblotting assay. $^{*}p < 0.05$ versus control; $^{**}p < 0.001$ versus control. B = BBP; C = control; D = DBP; H = DEHP.

Phthalate exposure assessment in humans has been chiefly conducted through the biomonitoring of phthalate metabolites in urine. Thus, we are not able to assess the actual serum concentration of those who have been exposed to phthalates. However, we need to take into account that the concentration of phthalates used for evaluation in the present study (10nM) is lower than the recommended daily exposure. Therefore, the biologic effects of phthalates should not be ignored or misconstrued as insignificant solely because of their low concentrations in the human body or their lower potency as compared with endogenous estrogens. Because the combinative effects of E_2 and phthalates even at very low concentrations is a potential cause of concern, further evaluation to redefine the recent reference doses for phthalates is imperative.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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