Synergistic effects of octylphenol and isobutyl paraben on the expression of calbindin-D_{9k} in GH3 rat pituitary cells

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Abstract. Endocrine disruptors (EDs) have estrogenic activity and can cause physiological estrogenic responses. Octylphenol (OP) is one of the alkylphenolic compounds known as environmental xenoestrogens because they strongly compete with endogenous estrogens to bind to estrogen receptors (ERs). Isobutyl paraben (IBP), a widely used preservative, also exhibits estrogenic activity. Calbindin-D_{9k} (CaBP-9k) is a novel biomarker for the detection of EDs used in our previous studies. In this study, the *CaBP-9k* gene was utilized as a marker for the estrogenic activity of combined OP and IBP to investigate possible additive, synergistic or antagonistic effects of these compounds in GH3 rat pituitary cells. GH3 cells were treated with different individual or combined doses of OP and IBP. In addition, the antiestrogen ICI 182,780 was used to examine the potential involvement of ERs in the induction of *CaBP-9k* expression by EDs. It was found that *CaBP-9k* expression was significantly increased at a high-dose of OP (1 μM) combined with each dose of IBP (0.1, 1 or 10 μM) compared to all single doses of IBP and OP. A synergistic increase in luciferase activity and *CaBP-9k* expression was observed following combination treatment with OP and IBP. Expression of the progesterone receptor (PR) gene was similarly induced by combined treatment with OP and IBP. In addition, pre-treatment with ICI 182,780, an estrogen antagonist, significantly blunted ED-induced *CaBP-9k* and *PR* expression. In summary, the expression of *CaBP-9k* and *PR* was induced more potently by combined OP and IBP than by treatment with either ED alone. ICI 182,780 treatment reversed ED-induced *CaBP-9k* and *PR* expression in these cells. Taken together, these results indicate that combined exposure to OP and IBP has a synergistic effect on the induction of *CaBP-9k* and *PR* gene expression via an ER-dependent pathway in GH3 cells.

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Introduction

Endocrine disruptors (EDs) influence hormone regulation, disrupting the function of the endocrine system in ways that can cause adverse health effects in organisms or their progeny. Some EDs occur naturally, such as the antioxidant flavonoids, which are found in fruits and vegetables, and others are industrial chemicals, such as some types of plasticizers, which can act as environmental contaminants. A wide range of species, from crustaceans, fish and birds to humans and other mammals, have been reported to be dysregulated by EDs, although the health significance of EDs exposure in human beings is still unclear (1). Humans and wildlife are exposed to a mixture of EDs, a point of significant interest, not the least because of the continuing discovery of new chemicals with endocrine-disrupting potential and the potential health effects of exposure to a multitude of chemicals simultaneously, rather than to single agents. In studying EDs mixtures, many researchers have followed a 'whole mixture approach' in which a combination of many chemicals is investigated as if it were a single agent but the individual effects of each of the components are not assessed. Whole mixture approaches do not address whether chemicals act in an additive, antagonistic or synergistic fashion. However, one of the major difficulties in studying EDs is uncertainty about their potential to act together in an additive or synergistic manner (2-4). In addition, humans are exposed to single EDs at low levels and EDs have much lower potent biological effects than natural hormones. However, humans are exposed to mixtures of EDs, and their combined effects should be studied to evaluate human exposure risk (5).

The estrogen receptor (ER) is a member of the steroid/thyroid hormone nuclear receptor superfamily and acts as a ligand-activated transcription factor. The ER has two forms, ERα and ERβ, which share common structural and functional characteristics but are encoded by separate genes located on different chromosomes. ERα is expressed in the uterus, mammary gland, testis, pituitary, liver, kidney and heart (6). ERβ is found in the prostate and ovaries (7-9). The ER subtypes are differentially involved in the induction of pituitary calbindin-D_{9k} (CaBP-9k) in rats in response to ERα- and

ERβ-specific EDs. ER-dependent signaling is believed to contribute significantly to the enhanced expression of CaBP-9k and PR by specific EDs in the GH3 cell line and in immature rats (10-14).

Alkylphenolic compounds are the most widely used class of surfactants (15). Octylphenol (OP) is an alkylphenolic compound and a xenoestrogen. It strongly competes with native estrogen for binding to the ER. OP was shown to have estrogenic activity that is 1,000 to 10,000 less potent than that of 17β-estradiol (E2) (16,17). Parabens are used widely as preservatives in pharmaceuticals, cosmetic products, processed foods and beverages (18). Parabens are also xenoestrogens; they can bind to EDs and enhance their ER-dependent effects. Parabens were shown to induce an ER-dependent increase in gene expression in MCF-7 human breast cancer cells (19-21), the ZR-75-1 cell line (22,23), rodent models (13,24-28), fish (29), and GH3 cells (12). The estrogenic potency of parabens has been shown to depend on the length of their alkyl side chains (19,24). Estrogenicity of parabens appears to be distinct when compared to natural estrogen (20,30). Isobutyl paraben (IBP) has comparatively high estrogenic activity among parabens (31,32).

The xenoestrogen biomarker CaBP-9k is a cytosolic protein with high affinity for calcium (33). In the estrous cycle and in early pregnancy, CaBP-9k expression is respectively up- and down-regulated by E2 and progesterone in the rat uterus (34). E2 was shown to increase *CaBP-9k* gene expression in the rat pituitary gland. The rat GH3 cell line is a widely used pituitary somatolactotrophic cell line that is stimulated by estrogen and secretes prolactin (10,35,36). Therefore, the GH3 cell line is a good model for investigating the estrogenicity of EDs *in vitro*.

In the previous studies, an increase in *CaBP-9k* gene expression was observed in response to single ED administration *in vivo* and *in vitro* (10,11,13,37,38). However, the effects of combined ED administration on *CaBP-9k* expression *in vitro* are not well understood. Thus, we investigated the *in vitro* synergistic effects of two EDs, OP and IBP, on the induction of *CaBP-9k* expression in GH3 cells.

Materials and methods

Reagents and chemicals. 17β-estradiol (E2) and 4-tetra-octyl phenol (OP, minimum 90.0% purity) were purchased from the Sigma-Aldrich Company (Irvine, UK), and ICI 182,780 (also known as faslodex or fulvestrant) was purchased from Tocris (Ellisville, MO, USA). Isobutyl p-hydroxybenzoate (IBP, minimal 99.0% purity) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich Company) and stored as stock solutions at -20˚C. Rabbit anti-CaBP-9k and goat anti-rabbit antibodies were provided by Swant (Bellinzona, Switzerland). Anti-ERα and PR antibodies and horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and treatment. GH3 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; WelGene, Daegu, Korea), 100 IU/ml penicillin and 100 g/ml streptomycin (WelGene) at 37˚C in a humidified atmosphere of 5% CO_2. To treat GH3 cells with OP and IBP, cells were plated and grown to 70-80% confluence in 6-well plastic tissue culture dishes (Nunc™; Nunc, Roskilde, Denmark). To ensure the depletion of steroid hormones and growth factors in the cultures, the growth medium was replaced with starvation medium containing phenol red-free DMEM with 5% dextran-coated charcoal stripped FBS, 100 IU/ml penicillin and 100 g/ml streptomycin, as described previously (37). GH3 cells were maintained in starvation medium for 7 days before exposure to different concentrations of OP (0.01, 0.1 or 1 μM) and/or IBP (0.1, 1 or 10 μM). Each chemical was dissolved in DMSO and added to the starvation medium with the final DMSO concentration being 0.1%. Starvation medium alone and 10^{-9} M E2 were used as controls. GH3 cells were harvested 24 h after treatment to measure mRNA and protein content. To examine the mechanism of *CaBP-9k* induction by EDs, cells were pretreated with 10^{-7} M ICI 182,780 (an antiestrogen) for 30 min prior to ED exposure. After ICI 182,780 treatment or no ICI 182,780 treatment, cells were treated with a high-dose of OP (1 μM) and 0.1, 1 or 10 μM IBP. The concentrations of OP and IBP used were those that produced the highest responses in GH3 cells in the concentration-response experiment. After 24 h, whole cells were harvested for mRNA and Western blot analysis. All experiments were performed in triplicate.

Quantitative real-time PCR. Total-RNA was extracted using the TRI reagent (Ambion, Austin, TX, USA) according to the methods outlined in the protocol, and the concentration of total-RNA was determined by measuring the absorbance at 260 nm. One microgram of a total-RNA was reverse transcribed into first-strand cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and a 9-mer random primer (Takara Bio, Inc., Shiga, Japan). RT-PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations. The relative expression level of each gene was normalized to that of cytochrome c oxidase subunit 1 (an internal control gene; 1A) and quantified using RQ software (Applied Biosystems). Quantitative real-time PCR reactions were performed using 1 μl of cDNA template added to 10 μl of 2X SYBR Premix Ex Taq (Takara Bio, Inc.) containing specific primers at a concentration of 10 pM each. Reactions were carried out for 40 cycles. The cycling parameters were as follows: denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all the samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity threshold in the exponential phase of PCR amplification was taken to be the threshold cycle (CT). The PCR product of *1A* was used as a control for variations in mRNA concentrations in the real-time PCR reactions. The primer sequences for *1A* were 5'-CCA GGG TTT GGA ATT ATT TC-3' (sense) and 5'-GAA GAT AAA CCC TAA GGC TC-3' (antisense). The primer sequences for *CaBP-9k* were 5'-AAG AGC ATT TTT CAA AAA TA-3' (sense) and 5'-GTC TCA GAA TTT GCT TTA TT-3' (antisense). The primer sequences for *PR* were

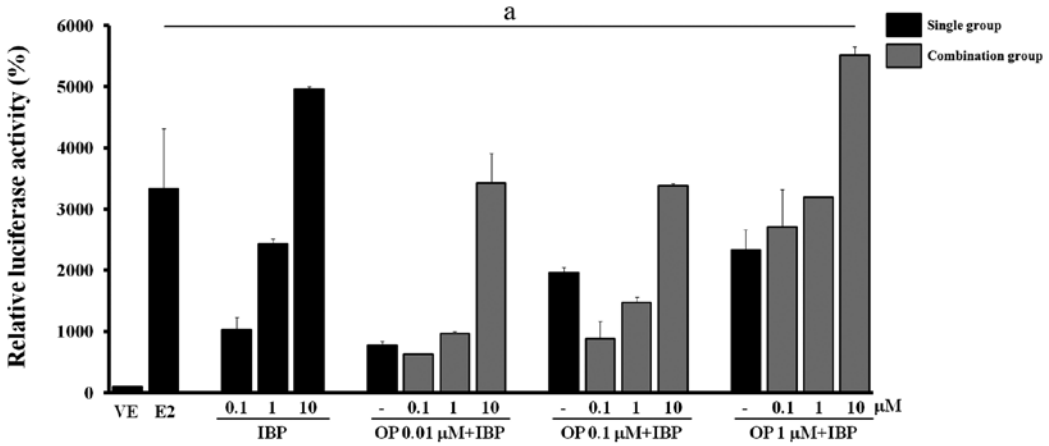

Figure 1. Effects of single or combination treatment with OP and IBP on ERE activity. As a negative control, GH3 cells transfected with p(ERE)3 constructs were treated with DMSO alone. As a positive control, cells were treated with E2 (1 nM) as indicated. GH3 cells were treated with OP (0.01, 0.1 or 1 μM) and/or IBP (0.1, 1 or 10 μM) and harvested 24 h after treatment. An expression vector encoding RSV-*lacZ* was co-transfected to allow normalization for transfection efficiency. Luciferase activity is represented as percent induction after normalization to β-galactosidase compared to cells transfected with the pGL3-promoter, which was set as 100%. Data represent the means ± SEMs of triplicate samples. [a]P<0.05 compared with a vehicle (VE).

5'-CAC AGG AGT TTG TCA AGG TC-3' (sense) and 5'-GGG ATT GGA TGA ACG TAT TC-3' (antisense).

Western blot analysis. Protein samples were extracted with PRO-PREP solution (Intron Biotechnology, Seoul, Korea) following the manufacturer's protocol. Thirty microgram of cytosolic protein per lane was size-fractionated in 7.5 and 12.5% tris-glycine and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes were then blocked for 60 min with 5% skim milk (Difco, Sparks, MD, USA) in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). Primary and secondary antibodies were incubated with the membranes in 5% skim milk in TBS-T for 1 h at room temperature. Antibodies against rat CaBP-9k (diluted 1:2,000, CB9, Swant) and PR (diluted 1:500, sc-538, Santa Cruz Biotechnology, Inc.) were utilized. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (diluted 1:3,000) and Western blotting luminol reagent (Santa Cruz Biotechnology, Inc.) were used to assess immunoreactivity. Each immunoblot was stripped with 2% SDS and 100 mM mercaptoethanol in 62.5 mM Tris-HCl pH 6.8 for 30 min at 50-60˚C and then membranes were washed twice for 5 min in PBS-T, blocked for 1 h in 5% skim milk (39), and re-probed with antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (diluted 1:2,000, CSA-335, Assay Designs, Inc.; Ann Arbor, MI, USA). Immunoreactive proteins were visualized by exposure to X-ray film. Protein bands were quantified by image scanning, and optical density was measured using a Gel Doc EZ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after the data were corrected by background subtraction and normalized using GAPDH as an internal control.

Construction of reporter plasmid and transient transfection. Three copies of the ERE [p(ERE)^3] sequence were inserted into the pGL3-promoter vector (Promega, Madison, WI, USA) upstream of the SV40 promoter. ERE oligomers were synthesized to contain *Mlu*I and *Xho*I restriction sites at both termini. The ERE sequence was 5'-AGGTC ACTGT GACCC TGGGT CACTGT GACCC TGGGT CACTG TGACC-3'. For transient transfection, 3x10^5 cells/well were plated on a 6-well dish and transfected 18 h later with the ERE plasmid by lipofection using Lipofectamine™ 2000 (Invitrogen Corporation) according to the manufacturer's directions. Each well was transfected using 4 μg of DNA and 10 μl of Lipofectamine™ 2000 reagent. The control plasmid RSV-*lacZ* (0.5 μg) (Clontech, Mountain View, CA, USA) was co-transfected to monitor transfection efficiency. After 4 h of incubation, the transfection mixtures were replaced with normal growth medium or hormone-supplemented medium. Following an additional 24 h of culture, cells were harvested and their luciferase activity was determined by a dual Luciferase assay (Promega). Luciferase activity was normalized for transfection efficiency after determining the β-galactosidase activity of the same sample. Each transfection was carried out in triplicate and experiments were repeated at least four times.

Statistical analyses. Results are presented as means ± standard errors of the mean (SEMs). P-values were calculated using one-way analysis of variance, followed by the Tukey test for multiple comparisons of columns. Data were considered statistically significant at P<0.05.

Results

Effects of single or combination OP and IBP treatment on ERE reporter gene expression. Transiently-transfected GH3 cells were incubated with a single or a combination treatment of OP and IBP for 24 h. An increase in luciferase activity was observed in response to E2 and single or combination treatments with OP and IBP (Fig. 1). Interestingly, luciferase activity was significantly increased by the highest dose of OP (1 μM) combined with each dose of IBP (0.1, 1 or 10 μM) compared to all single doses of IBP and OP. However, luciferase activity was significantly lower in cells treated with a lower dose of OP (0.01 or 0.1 μM) in combination with IBP (1 μM) than in those treated with a single dose of 1 μM IBP. Luciferase activity was also significantly lower in cells treated with a lower dose of OP (0.01 or 0.1 μM) in combination with IBP (10 μM) than in those treated with a single dose of 10 μM IBP.

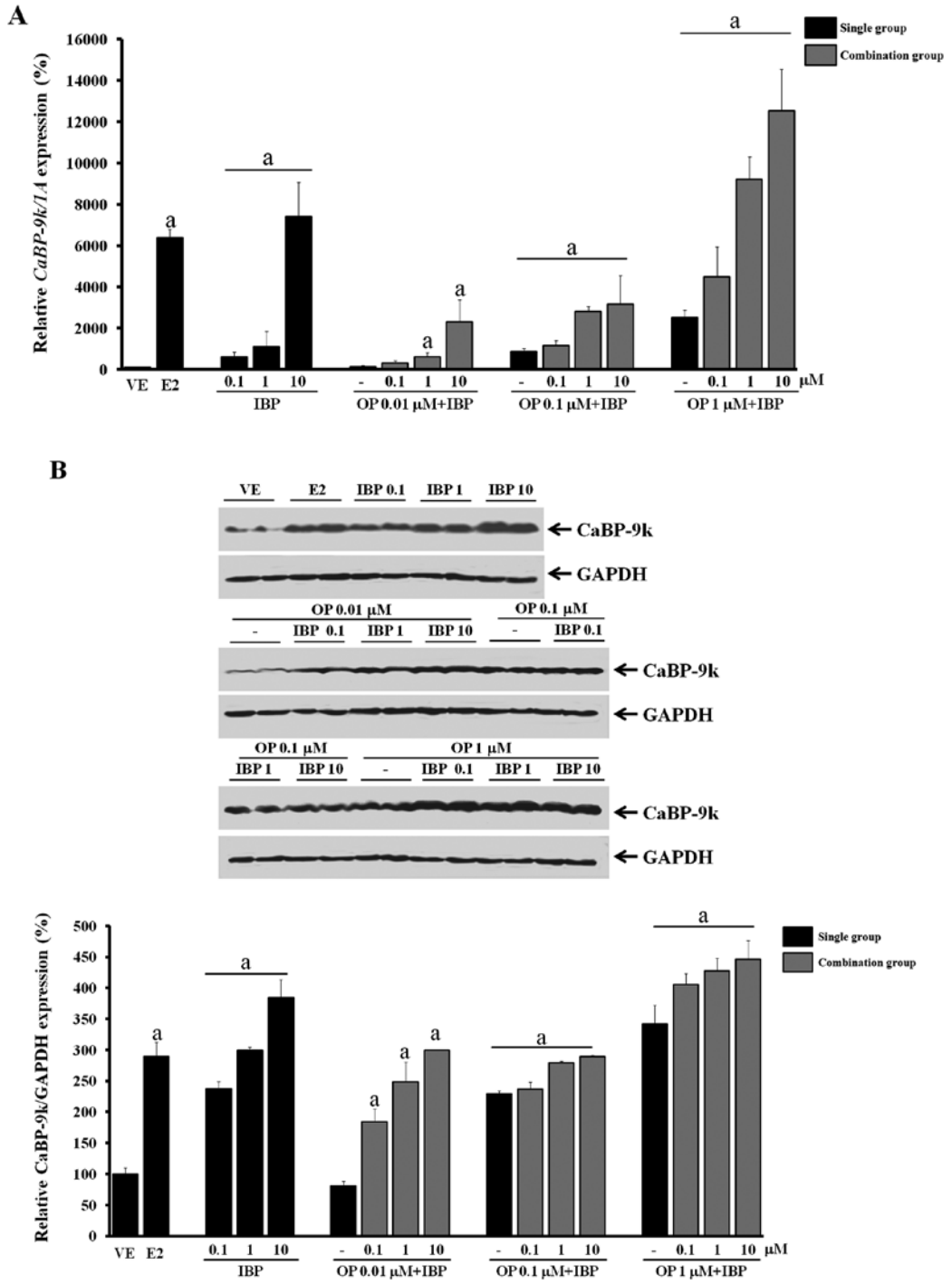

Figure 2. Effects of single or combination OP and IBP treatment on CaBP-9k expression. As a negative control, GH3 cells were steroid-deprived for 7 days in phenol red-free DMEM medium-FBS (5%) and treated with 0.1% DMSO alone (VE). As a positive control, cells were treated with E2 (1 nM) as indicated. Cells treated with OP (0.01, 0.1 or 1 μM) and IBP (0.1, 1 or 10 μM) were harvested 24 h after treatment. (A) *CaBP-9k* mRNA expression was determined by real-time PCR. (B) CaBP-9k protein expression was determined by Western blot analysis. *CaBP-9k* gene expression was normalized to that of internal controls (*1A* for mRNA and GAPDH for protein). Data represent the means ± SEMs of triplicate samples (mRNA) and duplicate samples (protein). [a]P<0.05 compared with VE.

Effects of single or combined OP and IBP treatment on the regulation of CaBP-9k and PR. Effects of single or combination OP and IBP treatment on the induction of *CaBP-9k* gene expression were examined using real-time PCR and Western blot analysis. Treatment with E2 or a single or combination treatment of OP and IBP resulted in a significant increase in the expression of *CaBP-9k* mRNA and protein (Fig. 2). Expression patterns of *CaBP-9k* mRNA induced by single or combined OP and IBP treatment were similar to those of CaBP-9k protein induced by single or combined OP and IBP treatment. Synergistic effects on *CaBP-9k* expression were observed with a high-dose (1 μM) OP treatment in combination with each dose of IBP (0.1, 1 or 10 μM). However, no synergistic effect on the induction of *CaBP-9k* was observed when cells were treated with 0.01 or 0.1 μM OP in combination with any dose of IBP. *PR* mRNA and protein expression were significantly induced by treatment

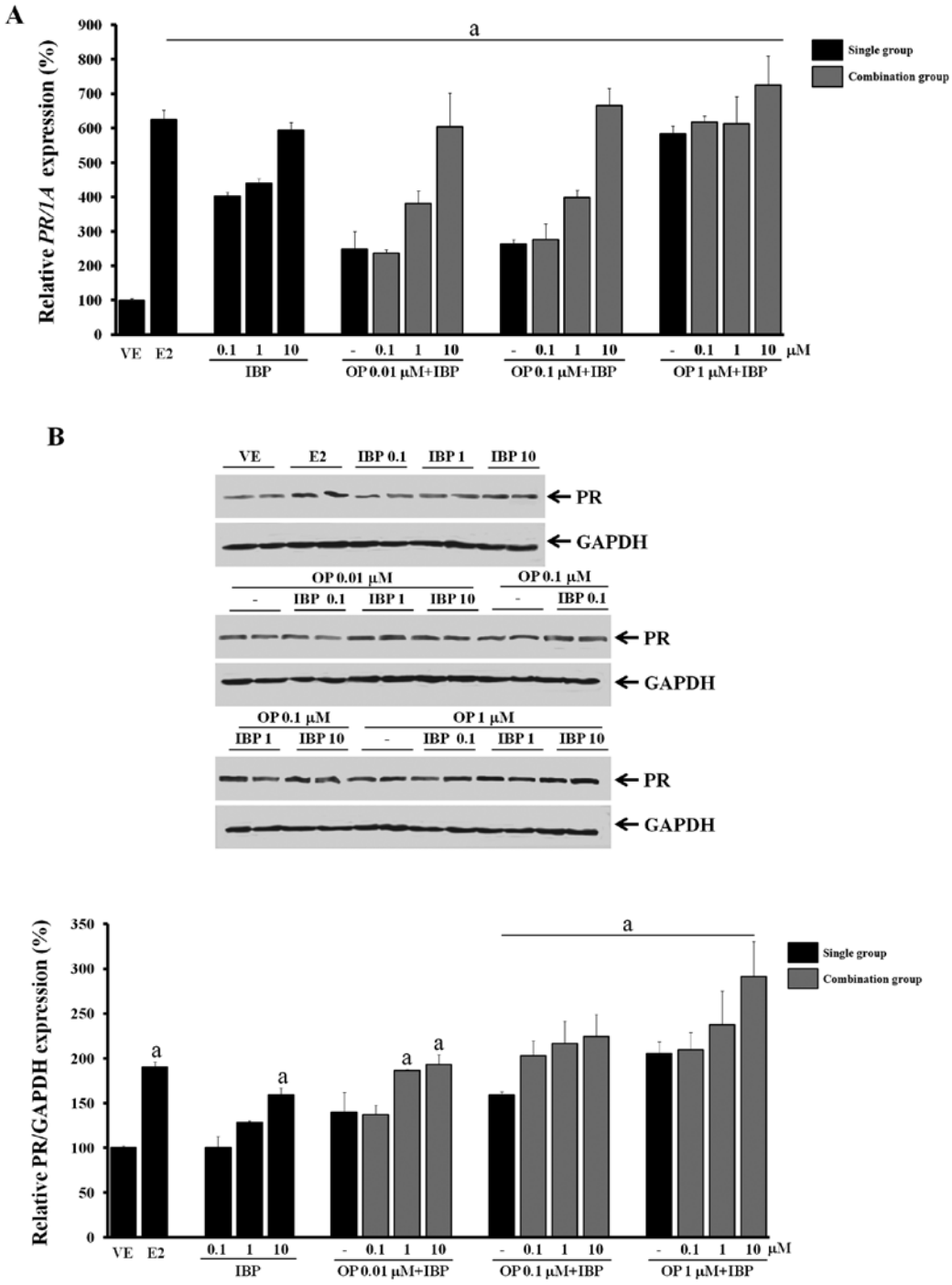

Figure 3. Effects of single and combined treatment with OP and IBP on PR expression. GH3 cells were steroid-deprived for 7 days in phenol red-free DMEM medium-CD FBS (5%) and treated with 0.1% DMSO alone (VE) as a negative control. As a positive control, cells were treated with E2 (1 nM) as indicated. Cells treated with OP (0.01, 0.1 or 1 μM) and IBP (0.1, 1 or 10 μM) were harvested 24 h after treatment. (A) *PR* mRNA expression was determined by real-time PCR. (B) PR protein expression was determined by Western blot analysis. *PR* expression was normalized to that of internal controls (*1A* for mRNA and GAPDH for protein). Data represent the means ± SEMs of triplicate samples (mRNA) and duplicate samples (protein). [a]P<0.05 compared with VE.

with E2 or a single or combined dose of OP and IBP (Fig. 3). A synergistic effect on *PR* expression was observed in response to a high-dose (1 μM) OP treatment in combination with any dose of IBP (0.1, 1 or 10 μM). Interestingly, the expression patterns of *PR* induced by single or combined doses of OP and IBP were similar to the patterns of *CaBP-9k*. In agreement with previous studies, the expression pattern of *CaBP-9k* and *PR* genes by OP and IBP appears to be similar to the enhanced expression of *CaBP-9k* and *PR* genes by other EDs.

Effects of antiestrogen on single or combined OP and IBP treatment on CaBP-9k and PR expression. To confirm that estrogen receptors are involved in the transcriptional and translational up-regulation of *CaBP-9k* and *PR* expression by a single or combination treatment with OP and IBP, we treated GH3 cells with ICI 182,780 before incubation with single doses of OP (1 μM) or IBP (0.1, 1 or 10 μM) or with a combination of OP (1 μM) and IBP (0.1, 1 or 10 μM). The induction of *CaBP-9k* and *PR* expression by a single dose of OP (1 μM) or

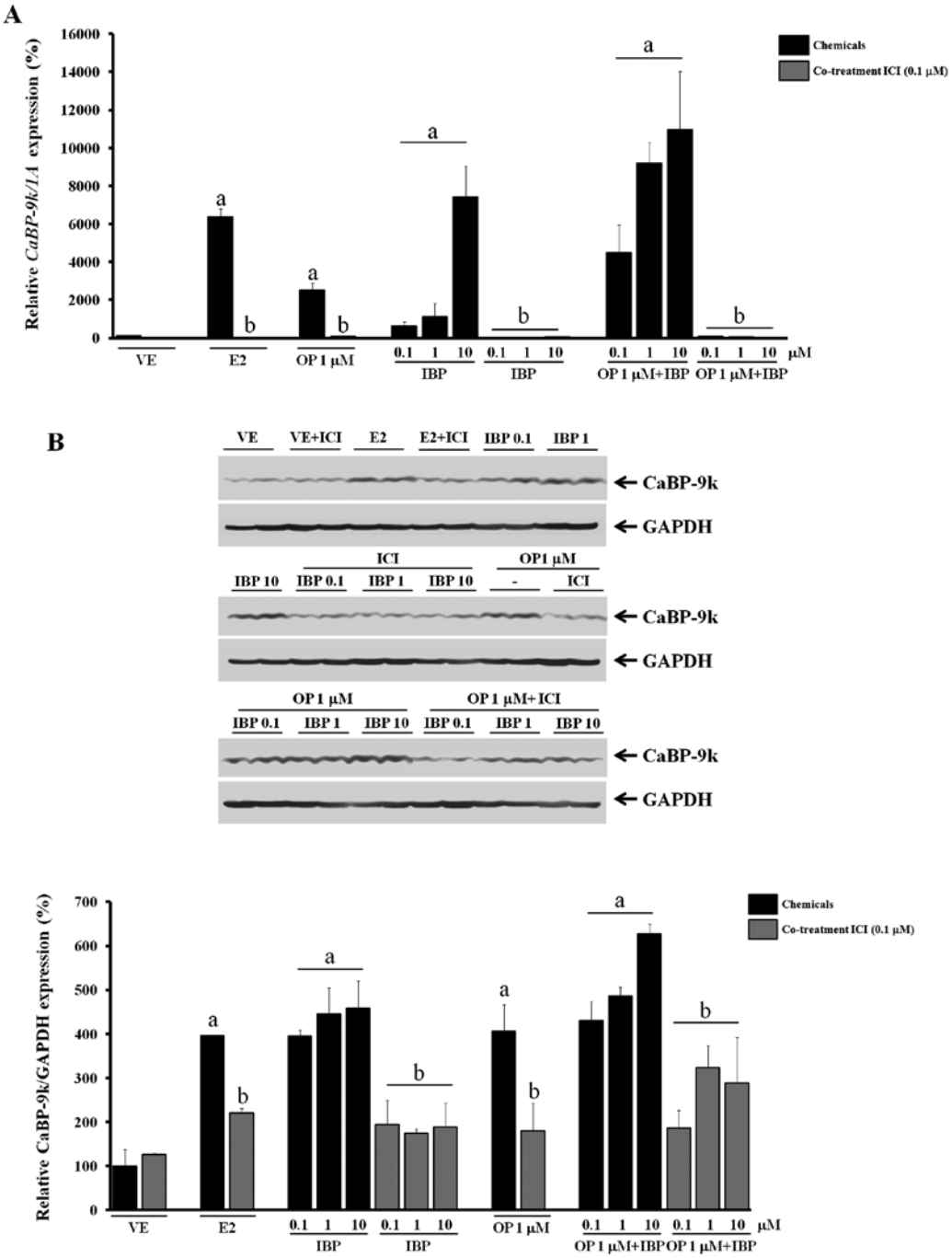

Figure 4. Effects of the antiestrogen ICI 182,780 on the expression of CaBP-9k mRNA and protein induced by single or combination OP and IBP treatment. GH3 cells were treated with DMSO alone, E2 (1 nM), OP (1 μM) or IBP (0.1, 1 or 10 μM), or with a combination of OP (1 μM) and IBP (0.1, 1 or 10 μM) in the presence or absence of 30 min pre-treatment with ICI 182,780 (0.1 μM). (A) *CaBP-9k* mRNA expression was determined by real-time PCR. (B) CaBP-9k protein expression was determined by Western blot analysis. CaBP-9k expression was normalized to that of internal controls (*1A* for mRNA and GAPDH for protein). Data represent the means ± SEMs of triplicate samples (mRNA) and duplicate samples (protein). [a]P<0.05 compared with VE; [b]P<0.05 compared with the single-treatment group.

IBP (0.1, 1 or 10 μM) or combination treatments of OP (1 μM) and IBP (0.1, 1 or 10 μM) was blunted by pre-treatment with ICI 182,780 (Figs. 4 and 5). These results indicate that the synergistic effect of combined OP and IBP on *CaBP-9k* and *PR* expression results from signaling through estrogen receptors.

Discussion

The present study investigated the possible synergistic effects of combined EDs. This study demonstrated that OP and IBP have estrogenic activity and act synergistically as detected by ERE luciferase activity and *CaBP-9k* and *PR* expression. This study also established that the effects of single or combined treatment with OP and IBP in the GH3 rat pituitary cancer cell line were mediated by ER signaling. Our previous study demonstrated that the *CaBP-9k* gene is a useful biomarker for the estrogenicity of EDs in GH3 cells (13,14,35-37,40).

In general, the activity of alkylphenolic compounds (APs) is highly dependent on the alkyl substitutions present in those compounds. A previous study compared APs with various

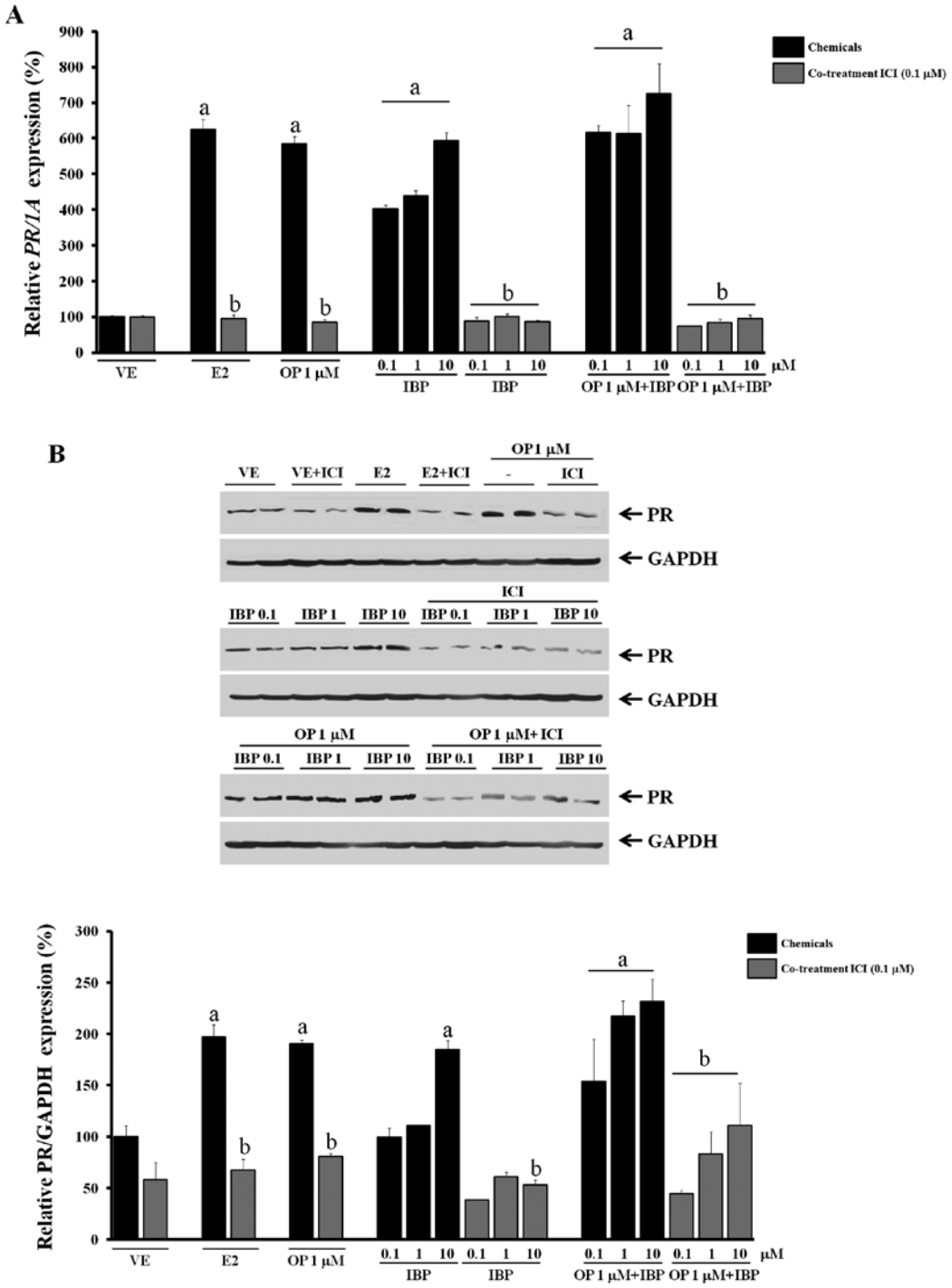

Figure 5. Effects of the antiestrogen ICI 182,780 on the expression of PR mRNA and protein. Cells were treated with DMSO alone, E2 (1 nM), OP (1 μM) or IBP (0.1, 1 or 10 μM), or with a combination of OP (1 μM) and IBP (0.1, 1 or 10 μM) in the presence or absence of 30 min pre-treatment with ICI 182,780 (0.1 μM). (A) *PR* mRNA expression was determined by real-time PCR. (B) PR protein expression was determined by Western blot analysis. PR expression was normalized to that of the internal controls (*1A* for mRNA and GAPDH for protein). Data represent the means ± SEMs of triplicate samples (mRNA) and duplicate samples (protein). [a]P<0.05 compared with VE; [b]P<0.05 compared with the single-treatment group.

alkyl groups, including propylphenol, butylphenol, NP, OP and phenylphenol, and found that, in a dot blot assay, *CaBP-9k* mRNA levels were increased in rats exposed to all APs. Also, a significant increase in uterine weight was observed in rats exposed to APs. Among APs, OP and nonylphenol (NP) showed the most potent effects. Both OP and NP contain bulk alkyl substitutions and have longer carbon chains than the other compounds examined (41). OP was also shown to increase *CaBP-9k* expression in GH3 cells (37). Thus, these results confirmed that OP has estrogenic activity of APs.

The other xenoestrogenic chemical tested in this study is a member of the paraben class of chemicals, which have structural similarities to APs. Parabens have weak estrogenic activity *in vitro* and *in vivo* (22,28,42,43). Long alkyl chain parabens (i.e., propyl-, isopropyl-, butyl- and isobutyl parabens) were shown to increase the expression of *CaBP-9k* in

GH3 cells (12). In addition, the binding affinity of parabens to ERα in MCF-7 cells is correlated with increasing alkyl chain length (20). Thus, as in APs, increased estrogenic activity is correlated with increased paraben side group alkyl chain length. The present study assessed the synergistic effects of combined OP and IBP treatment.

Several recent studies have examined the effects of combinations of EDs. One such study analyzed the combined estrogenic effects of a multi-component mixture of six plasticizers: bisphenol A (BPA), NP, OP, benzyl butyl phthalate, 4-chloro-3-methylphenol, and resorcinol. The mixture significantly induced ER activity in an additive manner in MVLN cells (44). Another study found that cell viability was decreased and LDH leakage was increased by a combination of NP and butyl phthalate in rat Sertoli cells (45). The combination of BPA and genistein (GEN) was shown to induce abnormal fetal and brain development in rat embryos and to increase adult rat uterine weight in a dose-dependent manner (4,46). Further, the combination of vinclozolin, an anti-androgenic food contaminant, and GEN showed a synergistic reduction of testosterone secretion and of gonocyte number (47). Therefore, combinations of EDs can induce toxicity and increase endocrine-disrupting effects.

CaBP-9k is a high affinity calcium-binding protein with two calcium-binding domains that is regulated by 17β-estradiol (E2) as well as by other steroid hormones, including 1,25-dihydroxyvitamin D3, glucocorticoids and progesterone. The *CaBP-9k* gene is a sensitive biomarker of the estrogenic potential of endocrine disruptors. *CaBP-9k* is expressed in duodenum, kidney, pituitary glands, and female reproductive tissues (34,48,49). Pituitary CaBP-9k expression is regulated during the estrous cycle. A significant increase in *CaBP-9k* expression is induced by E2 treatment in rats (36). In GH3 cells, it is well documented that estrogen activates *CaBP-9k* expression through an estrogen-responsive element (ERE) located in the 5'-upstream regulatory region of the gene (50). We recently showed that treatment with individual EDs including OP, NP and BPA, or parabens (methyl-, ethyl-, propyl-, isopropyl-, butyl- or isobutyl paraben) resulted in an increase in *CaBP-9k* expression (12,37). The present study further demonstrated a synergistic effect of combined EDs in GH3 cells. A high dose of OP (1 μM) in combination with each dose of IBP (0.1, 1 or 10 μM) showed synergistic effects on the induction of *CaBP-9k* and *PR* expression in GH3 cells. The patterns of *PR* and *CaBP-9k* expression induced by combined treatment with OP and IBP were similar. No synergistic effects were observed on the induction of *CaBP-9k* and *PR* in response to combination treatment with either of the two lower doses of OP (0.01 or 0.1 μM) with any dose of IBP. Similar results were shown by Jin *et al* (51) and Jukosky *et al* (52). In addition to the effects on *CaBP-9k* and *PR* expression, luciferase activity was induced by combined OP and IBP treatment in cells transfected with an ERE construct. When cells were pre-treated with an estrogen receptor antagonist, ICI 182,780, prior to treatment with single or combined OP and IBP, the estrogenic effects of these EDs were attenuated, suggesting that OP and IBP induce *CaBP-9k* and *PR* expression via an ER-mediated physiological pathway. Future studies are required to gain a deeper understanding of the mechanism of the synergistic effects of EDs.

In conclusion, people are typically exposed to combinations of ED in their daily life and environment. However, the estrogenic activity of EDs has traditionally been assessed by studying the effects of individual EDs. Here, we tested the combined and individual estrogenic activity of two EDs, OP and IBP, in GH3 cells. This study demonstrated a synergic effect of the combined EDs as detected by ERE luciferase activity and *CaBP-9k* and *PR* expression. We also found that these effects were blunted by pre-treatment with an ER antagonist, suggesting that this process is mediated by an ER-dependent pathway. Taken together, these results emphasize the importance of considering the combined effects of ED in assessing their risk to human health.

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