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Original articles

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ADDITIONAL EFFECTS OF BISPHENOL A AND PARABEN ON THE INDUCTION OF CALBINDIN-D_{9K} AND PROGESTERONE RECEPTOR VIA AN ESTROGEN RECEPTOR PATHWAY IN RAT PITUITARY GH3 CELLS

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There are concerns about the combined estrogenic effects of chemicals since mixtures of these chemicals exist in our environment. This study investigated potential additional interactions between bisphenol A (BPA) and isobutylparaben (IBP), which are major xenoestrogens used in the manufacture of plastics, cosmetics, drugs, and other products. The combined effects of these two chemicals were analyzed by measuring the expression of calbindin-D_{9k} (CaBP-9k) in rat pituitary cancer GH3 cells. GH3 cells were treated with single and combination doses of both chemicals (BPA single doses: 10^{-7}, 10^{-6} and 10^{-5} M; IBP single doses: 10^{-7}, 10^{-6} and 10^{-5} M, and each of the BPA and IBP doses combined). Prior to treatment, cells were temporarily transfected with a plasmid containing an ERE-luciferase reporter gene. Luciferase activity was measured as an indicator of ER activation by 17β-estradiol (E2), BPA, and IBP. BPA (10^{-5} M) combined with IBP (10^{-7} M and 10^{-6} M) induced a significant increase in the luciferase activity. Twenty-four hours after treatment, dose-dependent effects were observed in both single and combined dose groups, and several combination doses induced significant increases in the expression of CaBP-9k and progesterone receptor (PR) at both transcriptional and translational levels. Pre-treatment with ICI 182,780, a pure estrogen antagonist, significantly reversed BPA- and IBP-induced CaBP-9k and PR upregulation in GH3 cells. Taken together, these results indicate that BPA and IBP may have additionally increased estrogenic potency *via* an estrogen receptor-mediated pathway.

Key words: *progesterone receptor, estrogen receptor, 17β-estradiol, bisphenol A, calbindin-D_{9k}, isobutylparaben, cancer, GH3 cells*

INTRODUCTION

Many environmental agents are natural or synthetic chemicals with significant effects on the endocrine system. Chemicals that have harmful effects on the endocrine system are called endocrine disrupting chemicals (EDCs). EDCs induce their effects through diverse mechanisms, including binding to steroid hormone receptors and direct effects on cell signaling pathways (1). Numerous EDCs are widely used in commercial products regulated by the Food and Drug Administration (FDA), including food packaging materials, food additive, cosmetics, pharmaceuticals, and medical devices. Concerns raised by researchers regarding the possible effects of these chemicals on wildlife and human populations highlight the need for better understanding of the effects of EDCs. Although there is evidence to suggest that many of these chemicals cause adverse effects in humans, attempts to establish a relationship between human health risks and exposure to xenoestrogens are complicated by the fact that many of these chemicals have very mild estrogenic effects compared to 17β-estradiol (E2) (2). Therefore, it is difficult to account for risks arising from the toxicity of single chemicals. However, human and animals can be exposed to complex mixtures of EDCs, and it is possible that these chemicals might interact with each other in a way that increases or decreases their estrogenicity. This has motivated the analysis of possible combined estrogenic effects of environmental chemicals (3). More complex interactions may occur if two chemicals act on distinct but related targets. In extreme cases, there may be synergistic effects, in which the effects of two agents are greater than addition of their individual effects (4).

In the present study, we sought to reveal the potential additional effects of two EDCs: bisphenol A (BPA) and isobutylparaben (IBP). BPA is produced worldwide for use in a variety of industrial and consumer products, such as epoxy resins used to line food cans, polyester-styrene, and polycarbonate plastics, which are used in some baby bottles and other containers (5). BPA in food and beverage packaging is likely the source of most human oral exposure, and dermal and inhaled exposure may occur from other sources (6). Biochemical assays have investigated the kinetics of BPA binding to estrogen receptors (ERs) and have found that BPA binds to both estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) (7, 8). The affinity of BPA for ERs is 1,000–10,000-fold weaker than that of E2. BPA has been regarded as a very weak xenoestrogen

due to its low ER affinity. However, recent studies of the molecular mechanisms of BPA action have revealed a variety of pathways through which BPA can stimulate cellular responses at very low concentrations (9). Thus, adverse effects of BPA on human health are possible. BPA mimicked E2 in inducing prolactin expression and release and cell proliferation in both primary anterior pituitary cells and GH3 cells. Similar effects were observed in MCF-7 cells, and BPA was shown to induce estrogenic changes in the uterus of the CD-1 mouse (10, 11).

Parabens are widely used as preservatives in cosmetics, foods and drugs (12, 13). The estrogenicity of these compounds has been examined *in vitro* and *in vivo*. In some screening tests, parabens showed estrogenic activity, such as ligand binding to ERs and the proliferation of MCF-7 cells, and the reported *in vivo* effects include increased uterine weight and male reproductive tract effects (14, 15). Isobutylparaben (IBP) has relatively high estrogenic activity among the parabens (16). Like other EDCs, IBP can bind to ERs, stimulating an ER-dependent response and influencing the expression of estrogen-responsive genes, including ERα and PR (17).

We have chosen BPA and IBP among EDCs since they are both estrogenic. In addition, human have more opportunity to be exposed to these chemicals from many plastic wares and cosmetic products (18, 19). To demonstrate the combined effects of BPA and IBP, we designed an *in vitro* experiment using the calbindin-D_{9k} (CaBP-9k) gene as a biomarker induced by xenoestrogen exposure (20, 21). Other recent studies have also used reliable biomarkers to evaluate and characterize the estrogenicity of EDCs. The potency of EDCs was determined by assays monitoring the response of biomarkers to EDC exposure, illuminating the additional effects of these two chemicals (17, 22-24).

CaBP-9k is a member of large family of intracellular calcium binding proteins that have high affinity for calcium. CaBP-9k has been proposed as a new biomarker to detect EDCs. CaBP-9k has been shown to be expressed in several mammalian tissues, including kidney, uterus, and intestine (25-30). The estrogen responsive element (ERE) and progesterone responsive element (PRE) are present in the CaBP-9k promoter and mediate transcriptional regulation of CaBP-9k in the rat uterus (31). The ERE in the rat CaBP-9k gene is located at nucleotide +51 (transcriptional initiation site = +1) (32) and oligonucleotides containing the minimal ERE for the CaBP-9k promoter (nucleotides +51 to +61) is able to bind to ERα (33).

The GH3 cell line is a well-established pituitary cell line sensitive to estrogenic stimulation (34) and dependent on estrogen for proliferation in culture (35). We employed GH3 cells in this study to examine additional effects of BPA and IBP, since GH3 cells express CaBP-9k gene regulated by E2 and EDCs (17, 36). In a previous study, we used GH3 cells as an *in vitro* model to examine the estrogenicity of parabens (17, 36). In the present study, GH3 cells were treated with various concentrations of BPA and IBP, and the transcription and translation of CaBP-9k and PR were analyzed by molecular techniques. In addition, we utilized ICI182,780 treatment to investigate the possible involvement of ERs in EDC-induced CaBP-9k and PR expression.

MATERIALS AND METHODS

Reagents and chemicals

17β-estradiol (E2) and bisphenol A (BPA) were purchased from Sigma Chemical Company (St. Louis, MO). Isobutyl p-hydroxybenzoate (IBP) (minimum 99.0% purity) was obtained from Tokyo Kasei Kogyo Co. LTD (Tokyo, Japan) and ICI182,780 (also known as flaslodex or fulvestrant) was purchased from Tocris (Ellisville, MO). All chemicals were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich Company, Ayrshire, UK) and stored as a stock solution at –20°C to avoid contamination. Rabbit CaBP-9k and goat-anti rabbit antibodies were provided by Swant (Bellsinzona, Switzerland). Anti-PR antibodies and horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

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), supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY) at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. GH3 cells were plated on 6-well plastic tissue culture dishes (NUNC™; Roskilde, Denmark) at a density of 3×10^6 cells/well and grown until 70–80% confluent. The media was replaced with phenol red-free DMEM supplemented with 5% charcoal dextran-stripped FBS and 100 U/ml penicillin-streptomycin for 7 days to ensure the depletion of steroid hormones in the cells. The cells used in these experiments were grown normally throughout the study. After 7 days, the cells were exposed to a single dose of BPA (at 10^{-7}, 10^{-6}, or 10^{-5}M), IBP (at 10^{-7}, 10^{-6}, or 10^{-5}M), or each combination of these doses. Each chemical was dissolved in DMSO and added to phenol red-free DMEM-5% FBS-CD (starvation media) with the final DMSO concentration being 0.1%. Starvation media with 10^{-9} M E2 was used as a positive control, and starvation media with DMSO only was used as a negative control (vehicle). GH3 cells were harvested 24 hours after treatment to measure mRNA and protein levels. To examine the mechanism of CaBP-9k induction by these EDCs, cells were pre-treated with 10^{-7} M ICI182,780 for 30 min prior to EDC exposure (37). After ICI182,780 treatment, cells were treated with high-dose BPA (10^{-5} M) in combination with 10^{-7}, 10^{-6} and 10^{-5}M IBP. These combination doses were administered both in the presence and in the absence of ICI182,780. The concentrations of BPA and IBP tested were those that produced the highest response in GH3 cells in a dose-response experiment. After 24 hours, whole cells were harvested for mRNA and Western blot analysis. All experiments were performed in triplicate.

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using 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 total RNA was reverse transcribed into first-strand cDNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 9-mer random primers (Takara Bio, Otsu, Shiga, Japan). Quantitative RT-PCR was performed using a real-time PCR system 7300 (Applied Biosystems, Foster City, CA, USA) with 1 μl of cDNA template added to 10 μl of 2× 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 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. 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 as the threshold cycle (CT). The PCR

product of cytochrome *c* oxidase subunit 1 (1A, a ubiquitously expressed housekeeping gene) (38) was used as a control for mRNA concentrations in the RT-PCR reactions. The relative expression level of each gene was quantified using RQ software (Applied Biosystems). The amount of transcript present was inversely related to the observed CT and, for every two-fold dilution in the amount of transcript, CT was expected to increase by 1. Relative expression (*R*) was calculated using the equation $R=2^{-[\Delta C \text{ sample-}\Delta CT \text{ control}]}$. To determine a normalized arbitrary value for each gene, every data point was normalized to the control gene (1A), as well as to the respective controls. The primers 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 primers 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 primers for PR were 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. Forty micrograms of cytosolic protein per lane was size-fractionated by 7.5% and 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membranes were then blocked for 2 hours with 5% skim milk (Difco™, Sparks, MD) in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). Primary and secondary antibodies were applied to the membranes in 5% skim milk in PBS-T for 1 hour each at room temperature. Antibodies were used against rat CaBP-9k (diluted 1:2000, CB9, Swant, Bellinzona, Switzerland) and PR (diluted 1:500, sc-538, Santa Cruz Biotech). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (diluted 1:3000) and Western blotting luminol reagent (Santa Cruz Biotechnology Inc., CA) 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; membranes were then washed in PBS-T (twice, for 5 min each), blocked for 1 hour in 5% skim milk (39), and re-probed with an antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (diluted 1:2000, CSA-335, Assay Designs Inc., Ann Arbor, MI). 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 EQ system (Bio-rad Laboratories Inc.) after the data were corrected by background subtraction and normalized using GAPDH as an internal control.

Construction of a reporter plasmid and transient transfection

Three copies of the ERE [p(ERE)^3] sequence were inserted into the pGL3-promoter vector (Promega, USA) upstream of the SV40 promoter. ERE oligomers were synthesized containing MluI and XhoI restriction sites at both termini. The ERE sequence was 5'-AGG TCA CTG TGA CCC TGG GTC ACT GTG ACC CTG GGT CAC TGT GAC C-3'. For transient transfection, 3×10^5 cells/well were plated onto a 6-well dish and transfected 18 hours later with a luciferase plasmid by transfection using lipofectamine™ 2000 (Invitrogen Corporation) according to the manufacturer's directions. Four micrograms of DNA and 10 µl of lipofectamine™ 2000 reagent were used per well. The control plasmid RSV-*lacZ* (0.5 µg) (Clontech, USA) was co-transfected to monitor transfection efficiency. After 4 hours of incubation, the transfection mixtures were removed and replaced with normal growth medium or hormone-supplemented medium. Following an additional 24 hours of culture, the cells were harvested and their luciferase activity was determined by a Dual Luciferase assay (Promega, USA). The activity was normalized for transfection efficiency after determining the β-galactosidase activity of each sample. Each transfection was carried out in triplicate, and experiments were repeated at least four times.

Statistical analyses

Results are presented as means ±standard error of the mean (S.E.M.); *p* values were calculated using one-way analysis of variance, followed by Tukey's test for multiple comparisons of columns. Data were considered statistically significant at $p<0.05$.

RESULTS

Effects of single or combined administration of bisphenol A and isobutylparaben on estrogen responsive element activity

Luciferase activity was induced by E2, BPA and IBP in GH3 cells transfected with the ERE-luciferase construct. Transiently transfected GH3 cells were incubated with single or combination doses of BPA and IBP for 24 hours. As shown in *Fig. 1*, increased luciferase activity was observed with both single and combination treatments (BPA single doses: 10^{-7}, 10^{-6} and 10^{-5} M; IBP single doses: 10^{-7}, 10^{-6} and 10^{-5} M; combined doses: 10^{-7}, 10^{-6} and 10^{-5} M for each) and was dose-dependent. In addition, luciferase activity was significantly higher with a combination of the highest dose of BPA (10^{-5} M) with the lowest (10^{-7} M) or middle dose of IBP (10^{-6} M) compared to a single dose exposure respectively. However, other combination doses did not induce a significant increase in luciferase activity, and the effects were masked with higher EDC doses.

Combined effects of bisphenol A and isobutylparaben on the expression of CaBP-9k mRNA and protein

The effects of BPA and IBP on the expression of CaBP-9k were assessed by RT-PCR and Western blot analysis. As shown in *Fig. 2*, dose-dependent effects were observed 24 hours after single or combined EDC exposure. A significant increase in the expression of *CaBP-9k* mRNA was observed with a mixture of the lowest concentration of BPA (10^{-7} M) and IBP (10^{-7} M) or the highest concentrations of BPA (10^{-5} M) and IBP (10^{-5} M), as seen in *Fig. 2A*. In addition, the expression of CaBP-9k protein was significantly increased with the lowest dose of BPA (10^{-7} M) and the highest dose of IBP (10^{-5} M), and with the highest dose of BPA (10^{-5} M) and IBP (10^{-5} M) combined compared to single exposures with these EDCs (*Fig. 2B*). Additional effects were not observed with other combined doses of these EDCs. Effects on CaBP-9k gene expression were masked with relatively higher doses. Expression of *CaBP-9k* mRNA was 462-fold higher and protein expression was 141-fold higher in the positive control (E2, 10^{-9} M) than a negative control. To determine the biological pathways involved in the regulation of CaBP-9k expression by combined BPA and IBP in GH3 cells, we pre-treated cells with ICI182,780. GH3 cells were treated with single or combination doses of BPA and IBP in the absence or presence of ICI182,780 (10^{-7} M) treatment 30 min prior to chemical exposure. As shown in *Fig. 3A*, ICI182,780 pre-treatment completely attenuated the transcription and translation of CaBP-9k induced by EDCs. This result indicates that the effects of BPA and IBP on the induction of CaBP-9k expression involve ER-mediated pathway in GH3 cells.

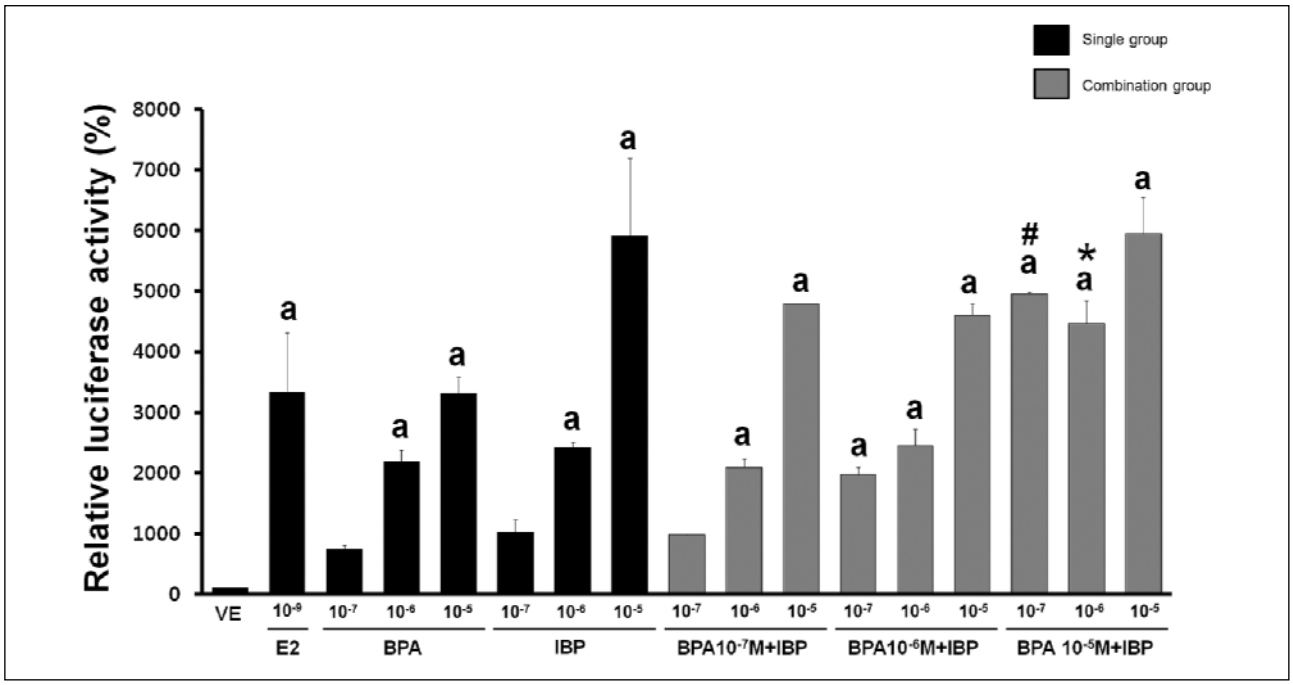

Fig. 1. Effects of single or combined treatment with BPA and IBP on ERE activity. GH3 cells transfected with p(ERE)^3 constructs were treated with 0.1% DMSO (VE) as a negative control, E2 at 10^{-9} M as a positive control, BPA alone (10^{-7}, 10^{-6}, and 10^{-5} M), IBP alone (10^{-7}, 10^{-6}, and 10^{-5} M), or a combination of BPA (10^{-7}, 10^{-6}, and 10^{-5} M) and IBP (10^{-7}, 10^{-6}, and 10^{-5} M). An expression vector encoding RSV-*lacZ* was co-transfected to normalize transfection efficacy. Luciferase activity is represented as percent induction after being normalized to β-galactosidase compared to cells transfected with pGL3-promoter, which was set as 100%. Data represent the means ±S.E.M. of triplicate experiments. a, $p<0.05$ compared to vehicle; #, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-7} M); *, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-6} M).

Combined effects of bisphenol A and isobutylparaben on the expression of progesterone receptor mRNA and protein in GH3 cells

We next assessed PR expression following single and combined treatment with BPA and IBP (BPA single doses: 10^{-7}, 10^{-6} and 10^{-5} M; IBP single doses: 10^{-7}, 10^{-6} and 10^{-5} M; combinations: each BPA dose combined with each IBP dose). After 24 hours, PR mRNA and protein expression had increased in a dose-dependent manner (*Fig. 4*). As shown in *Fig. 4A*, a strong increase in expression of *PR* mRNA was observed after combined treatment with the highest dose of BPA (10^{-5} M) and the middle dose of IBP (10^{-6} M) compared to a single exposure to each of these doses. The expression of PR protein, however, showed the greatest increase with a combination of the highest dose of BPA (10^{-5} M) with a middle (10^{-6} M) or highest dose (10^{-5} M) of IBP (*Fig. 4B*). Compared to a vehicle, the expression of *PR* mRNA was increased 189-fold, and protein expression was increased 26.4-fold by the positive control (E2, 10^{-9} M). No other additional effects on PR expression were observed.

To investigate whether the ER pathway mediates expression of PR, GH3 cells were treated with ICI182,780 30 min prior to EDC treatment. GH3 cells were treated with a single treatment of the highest dose of BPA (10^{-5} M), with IBP (10^{-7}, 10^{-6}, or 10^{-5} M), or with a combination of BPA (10^{-5} M) and IBP (10^{-7}, 10^{-6}, or 10^{-5} M). After 24 hours of EDC treatment, IBP was observed to up-regulate PR expression in a dose-dependent manner and this effect was completely abolished by ICI182,780 treatment (*Fig. 5*). These results provide evidence that BPA and IBP exposure in GH3 cells increases PR gene expression. In addition, these results further imply that an ER-mediated pathway is involved in the up-regulation of PR mRNA and protein.

DISCUSSION

EDCs exert their effects either by binding to hormone receptors or through direct action on cell signaling pathways and can have effects even at very low dose (40). Previous evidence suggests that the combined effects of EDCs from the same category, *i.e.*, estrogenic, anti-androgenic, or thyroid-disrupting agents, may act together through dose addition (41). The topic of combined exposure to EDCs has been considered important because environmental EDC exposure results from many chemicals and not from single chemicals. More complicated interactions may take place if two chemicals act on related targets and, in some cases, there may be additional effects (4).

We investigated the possible additional interaction of BPA and IBP. BPA and IBP are estrogenic agents that are able to evoke a response similar to that of E2, such as uterine cell proliferation (42). They appear to exhibit estrogenicity interacted with ERs resulting in the activation of estrogen-dependent gene expression (7, 8). BPA, known as an endocrine disruptor, is manufactured worldwide for use in various industrial and consumer products (5, 6). BPA is often used in food and beverage containers, including baby bottles, and is also used as an additional in other plastics (6). BPA was initially regarded as a weak xenoestrogen based on its relative affinity for the classical nuclear receptors ERα and ERβ which was estimated to be at least 1,000–10,000-fold lower than that of E2 (8). However, the latest studies have determined that BPA can stimulate cellular responses at very low concentrations. For instance, it was reported that BPA stimulated PKA and PKC pathways *via* a G-protein-coupled nonclassical membrane ER (GPER) at very low concentration (10^{-9} to 10^{-12} M) (43). Also, picomolar concentration of xenoestrogens including BPA has been known to trigger

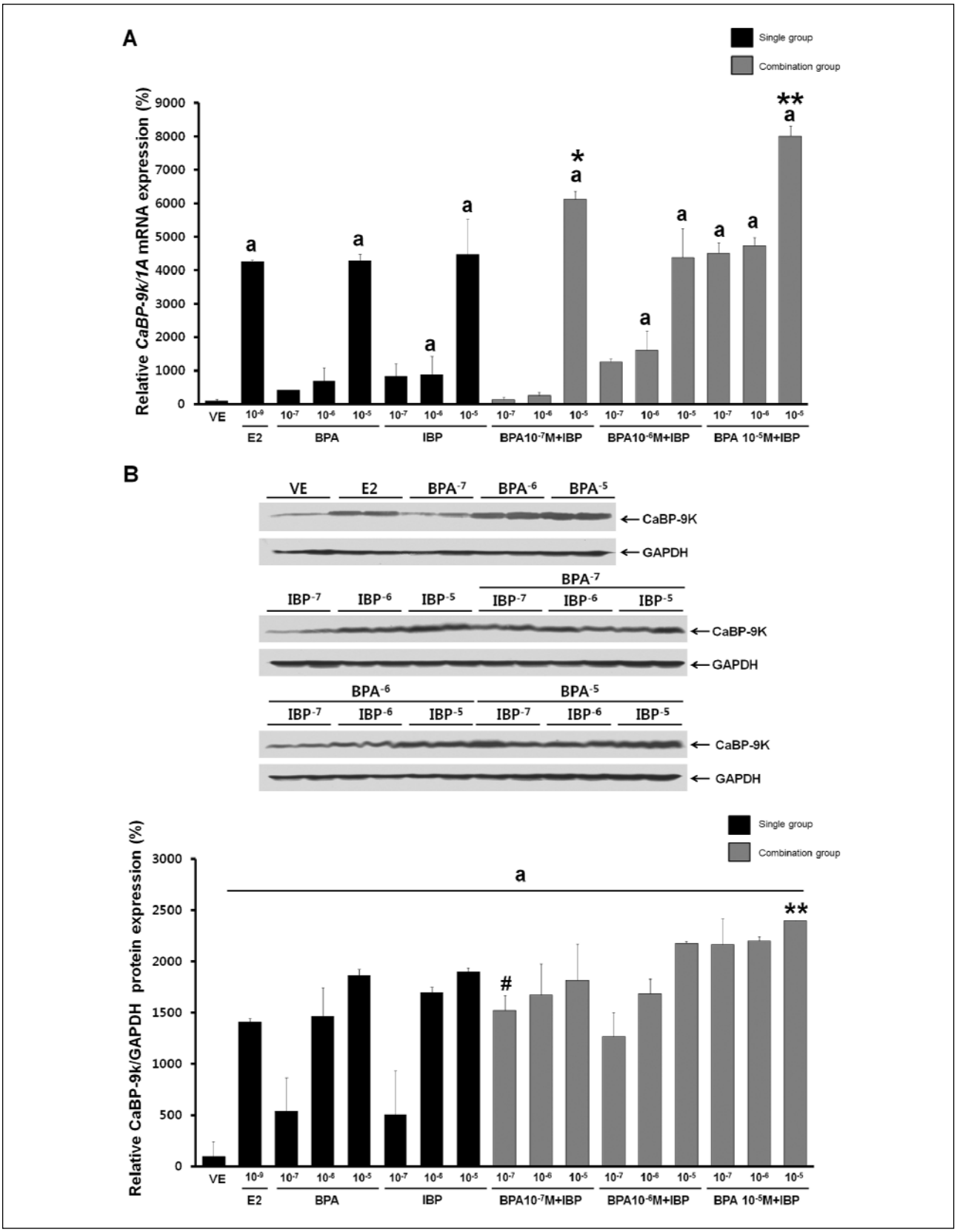

Fig. 2. Effects of single or combined BPA and IBP treatment on CaBP-9k mRNA and protein expression. Cells were treated with DMSO alone as a vehicle (VE); with E2 at 10^{-9} M as a positive control; or with BPA alone (10^{-7}, 10^{-6}, and 10^{-5} M), IBP alone (10^{-7}, 10^{-6}, and 10^{-5} M), or a combination of BPA (10^{-7}, 10^{-6}, and 10^{-5} M) and IBP (10^{-7}, 10^{-6}, and 10^{-5} M). (A) *CaBP-9k* mRNA expression was determined by RT-PCR. (B) CaBP-9k protein expression was determined by Western blot analysis. Data represent the means ±S.E.M. of triplicate experiments. CaBP-9k gene expression was normalized to that of an internal control gene (1A for mRNA and GAPDH for protein). a, $p<0.05$ compared to vehicle; *, $p<0.05$ compared to BPA alone (10^{-7} M) and IBP alone (10^{-5} M); **, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-5} M); #, p 0.05 compared to BPA alone (10^{-7} M) and IBP alone (10^{-7} M).

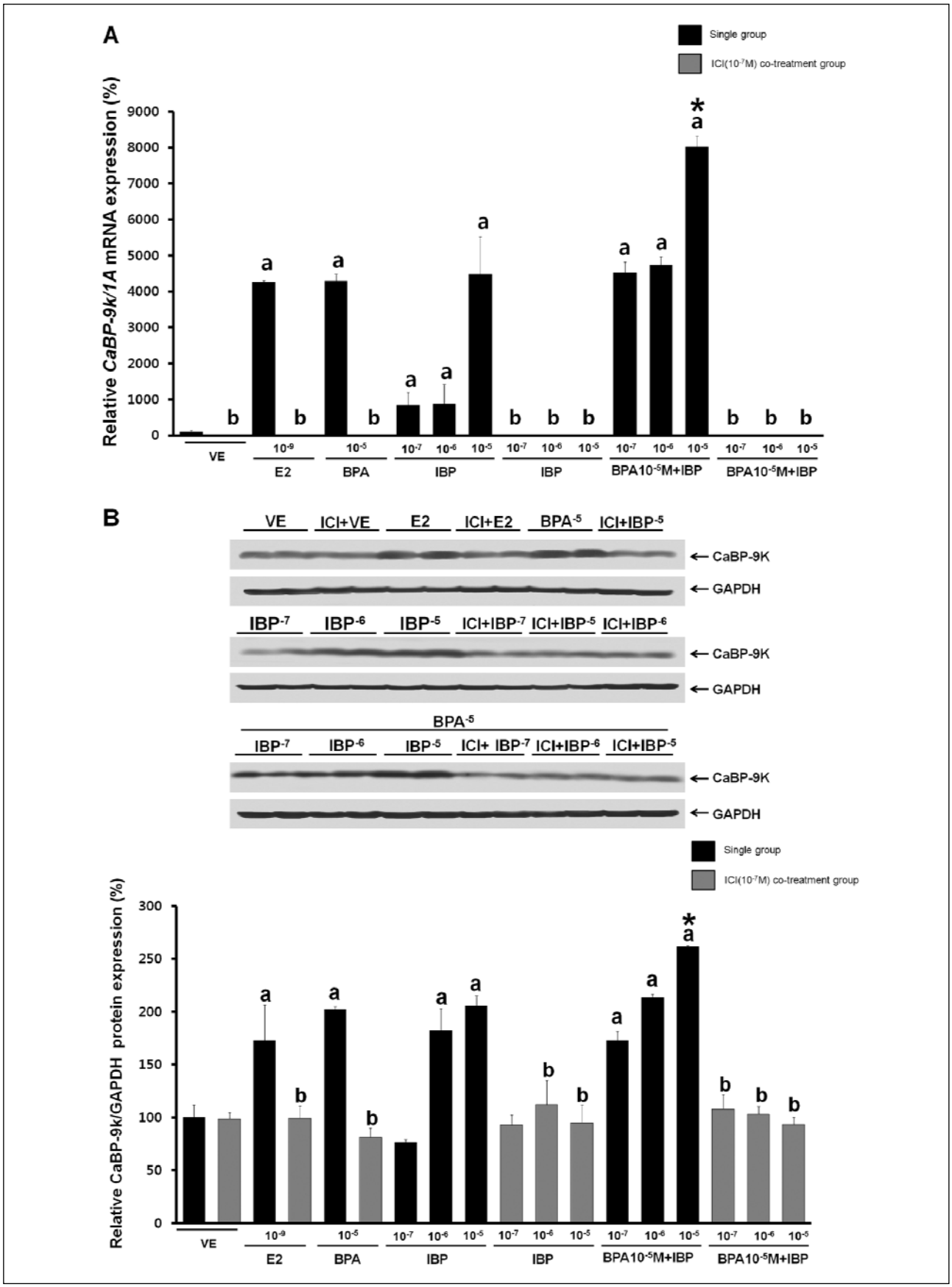

Fig. 3. Effects of ICI182,780 on the expression of CaBP-9k mRNA and protein. (A) Expression of *CaBP-9k* mRNA was determined by RT-PCR. (B) Expression of CaBP-9k protein was determined by Western blot analysis. Data represent the means ±S.E.M. of triplicate experiments. CaBP-9k expression was normalized to that of an internal control (1A for mRNA and GAPDH for protein). a, $p<0.05$ compared to vehicle; b, $p<0.05$ compared to EDC only; *, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP (10^{-5} M) alone.

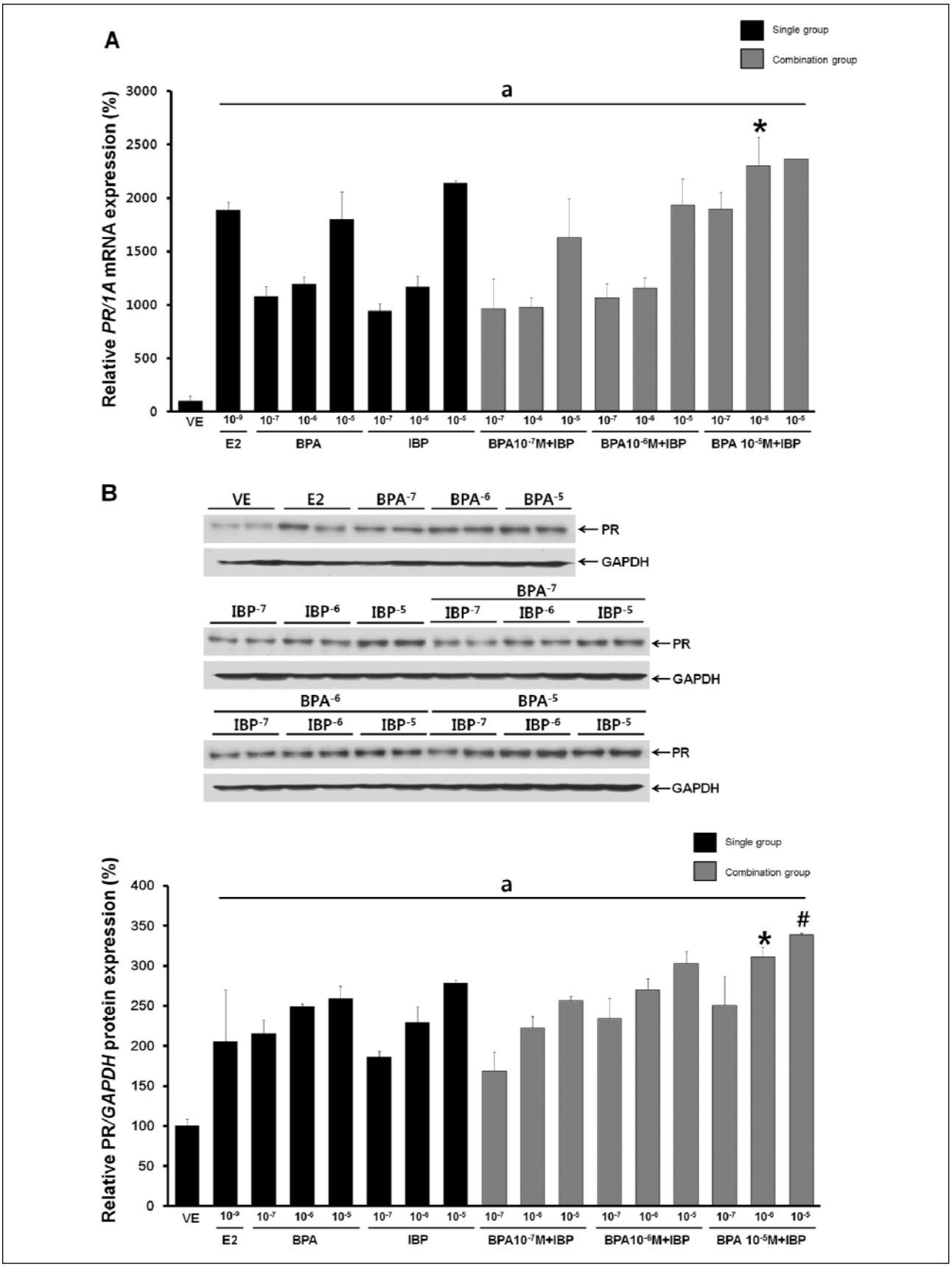

Fig. 4. Effects of single or combined BPA and IBP treatment on the regulation of PR mRNA and protein. (A) PR mRNA expression was determined by RT-PCR. (B) PR protein expression was determined by Western blot analysis. Data represent the means ±S.E.M. of triplicate experiments. PR expression was normalized to that of an internal control (1A for mRNA and GAPDH for protein). a, $p<0.05$ compared to vehicle; *, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-6} M); #, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-5} M).

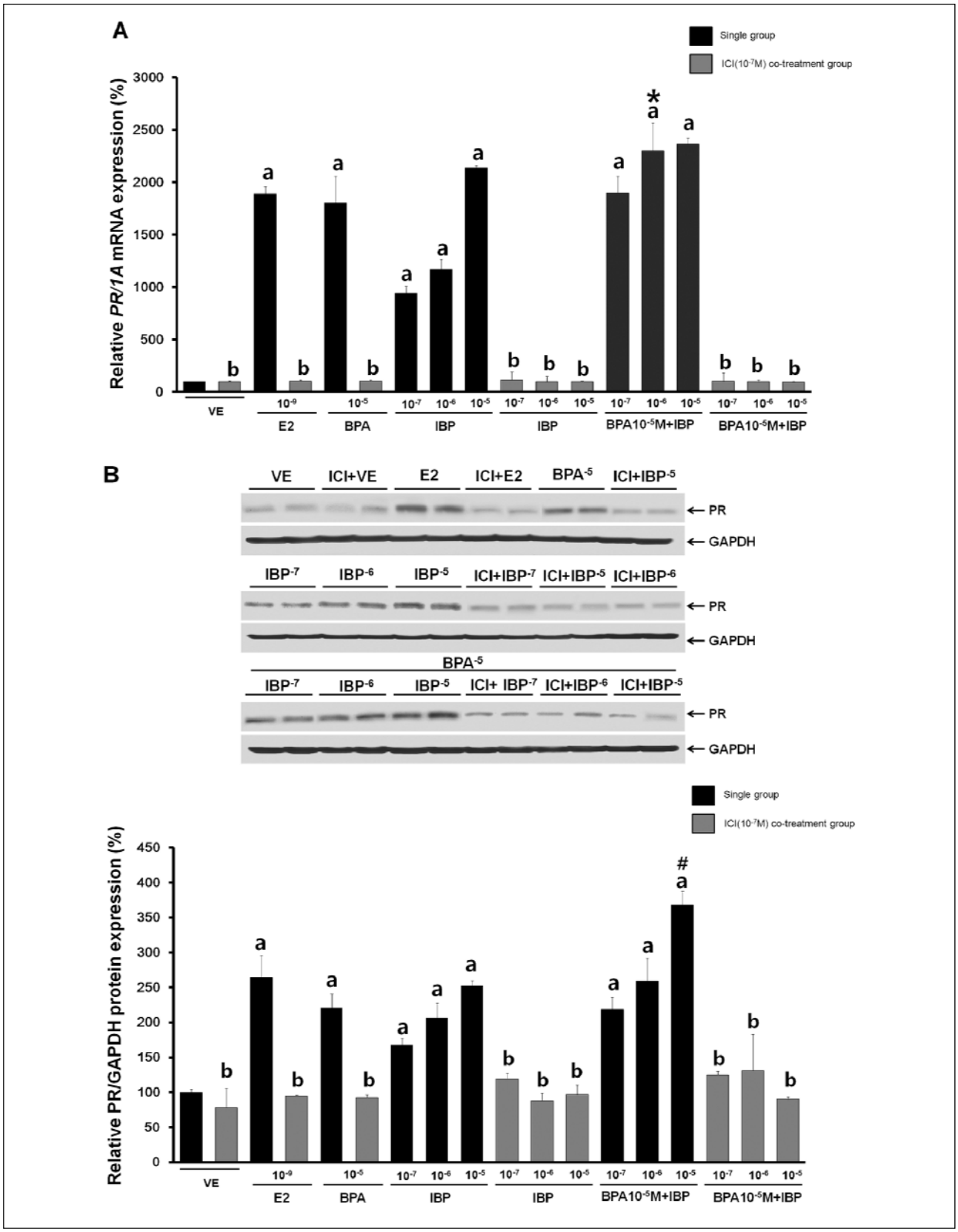

Fig. 5. Effects of ICI182,780 on the regulation of PR mRNA and protein expression. Cells were treated with DMSO alone as a vehicle (VE) or with E2 at 10^{-9} M as a positive control or with BPA alone (10^{-7}, 10^{-6}, and 10^{-5} M), IBP alone (10^{-7}, 10^{-6}, and 10^{-5} M), or a combination of BPA (10^{-7}, 10^{-6}, and 10^{-5} M) and IBP (10^{-7}, 10^{-6}, and 10^{-5} M) in the presence or absence of 30 min pretreatment with ICI182,780 (10^{-7} M). (A) Expression of *PR* mRNA was determined by RT-PCR. (B) Expression of PR protein was determined by Western blot analysis. Data represent the means ±S.E.M. of triplicate experiments. PR gene expression was normalized to that of an internal control (1A for mRNA and GAPDH for protein). a, $p<0.05$ compared to vehicle; b, $p<0.05$ compared to EDC only; *, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-6} M); #, $p<0.05$ compared to BPA alone (10^{-5} M) and IBP alone (10^{-5} M).

calcium influx (44) and ERK phosphorylation in rat pituitary cells (45). In some cases, BPA has been shown to be equivalent in potency to E2 in mice and rats (46, 47). Early BPA exposure has been reported to influence female reproduction, and altered estrous cycles, which have been reported in BPA-exposed females, can serve as the first indicator of disruption of the hypothalamic-pituitary-ovarian axis (48). BPA has been shown to affect the proliferation of estrogen-dependent MCF-7 cells in a dose-dependent manner, influence the proliferation of both primary anterior pituitary cells and GH3 cells, and induce estrogenic changes in the uterus of CD-1 mouse, similar to its action on MCF-7 cells (10, 11, 49, 50).

Parabens are widely used as preservatives in underarm cosmetics. Although parabens are generally considered safe, recent reports suggest that they show estrogenicity in a variety of *in vitro* tests, including the proliferation of MCF-7 and ZR-75-1 human breast cancer cell lines, and *in vivo* tests such as uterotrophic assays in both rat and mouse (15, 51-54). The estrogenic effect of parabens is approximately 1000 times less than that of E2, but parabens can bind to ERs stimulating an ER-dependent response (55). Parabens have effects on the regulation of estrogen response elements, including ERα and PR expression (17). Competitive binding assays utilizing human ERα and ERβ *in vitro* have suggested that parabens with longer side-chains have a greater affinity for estrogen receptors, but the relative binding affinity values were much lower than those of E2 (17, 56, 57). IBP has relatively high estrogenic activity among the parabens (16).

In this study, we used the CaBP-9k gene as a biomarker to evaluate the estrogenic potential of BPA and IBP (21, 25). Potency of single and combined EDCs was assessed using biomarker induction assays to reveal additional effects of the two chemicals. The ERE and PRE are located in the CaBP-9k promoter and mediate transcriptional regulation of CaBP-9k in the rat uterus (31). Interaction between the ERE and ER on the CaBP-9k gene promoter results in the regulation of gene expression, and ER mediates estrogen responsiveness in the pituitary cells (58). We investigated the effects of BPA and IBP on luciferase activity using a plasmid containing three copies of an ERE sequence to determine if ER-mediated pathways mediate EDC-induced expression of CaBP-9k and PR. We monitored transiently transfected cells with a plasmid containing ERE luciferase reporter gene. We showed that luciferase activity increased in a concentration-dependent manner following single or combined doses of BPA and IBP. Also, an additional effect on luciferase activity was observed with some combination doses, including a mixture of the highest dose of BPA (10^{-5} M) with the lowest dose of IBP (10^{-7} M). This finding implies that the estrogenic effects of BPA and IBP on CaBP-9k and PR gene expression are induced through ERs, and these EDCs have additional effects at certain doses.

Measurement of CaBP-9k mRNA and protein induced by BPA and IBP in GH3 cells showed dose-dependent up-regulation of CaBP-9k gene induction after 24 hours, similar to the effects of BPA and IBP on luciferase activity. Several doses of BPA and IBP showed probable additional effects on *CaBP-9k* mRNA and protein expression. Interestingly, CaBP-9k transcription and translation exhibited additional effects with the same combination dose (the highest dose of both BPA and IBP). We also observed that the expression of CaBP-9k was masked by relatively higher doses of BPA or IBP in the combined groups that did not show additional effect. For example, the expression of *CaBP-9k* mRNA induced by a combination of BPA (10^{-5} M) and IBP (10^{-6} M) was similar to that induced by a single dose of BPA (10^{-5} M). The lack of addition in CaBP-9k induction may be attributable to saturation of ER in these cells. On the other hand, high doses of hormonal agents revealed less responses than as expected, suggesting that its receptors may be down-regulated by the ligand (59).

To examine the biological pathway involved in the induction of CaBP-9k mRNA and protein, GH3 cells were pretreated with ICI182,780 30 min before EDC treatment. As expected, the estrogenic effects of BPA and IBP were diminished by pre-treatment with ICI182,780. This suggests that ER-mediated pathway is involved in the up-regulation of CaBP-9k expression by BPA and IBP. The PR gene is one of the most widely studied ER-regulated genes and its expression indicates functioning ER pathway (60). Therefore, we measured PR mRNA and protein expression after BPA and IBP exposure to better comprehend the mechanism of CaBP-9k induction by these estrogenic chemicals. Interestingly, the patterns of PR mRNA and protein expression were highly similar to those of CaBP-9k. Induction of PR expression increased in a dose-dependent manner, and additional effects were observed with some combination doses. ICI 182 780, an antagonist against ERs including ERα and ERβ, was employed to confirm the involvement of ER signaling while it acts as an agonist to GPER (61). GH3 cells were demonstrated to express these ERs (45). As anticipated, the effects of BPA and IBP were efficiently blocked by ICI182,780 pre-treatment.

In the present study, we demonstrated that the expression of the estrogenic biomarkers, CaBP-9k and PR gene, were additionally affected by combined EDCs. Furthermore, we showed that the combined estrogenic effect of BPA and IBP was mediated by ER-associated signaling pathway. These findings suggest that there are also possibilities of additive or synergistic influence of other EDCs in our body. Although the accumulated concentration of these chemicals are low, it is highly possible that combination of EDCs including BPA and IBP can bring more unanticipated severe adverse effects in the reproductive organs in both males and females. Because combination effects can result from agents present at or even below their effect thresholds, further analysis should be conducted to determine the concentrations needed for the additional effects of combined EDCs, and to understand the levels and characteristics of EDCs present in the environment and in human tissue.

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