

DNA METHYLATION OF ESTROGEN RECEPTOR α GENE BY PHTHALATES

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The phthalates are ubiquitous industrial plasticizers and include agents such as di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBP), which are classified as endocrine disruptors because of their anti-androgenic or pro-estrogenic effects. A recent study suggested that DBP produced the hypomethylation of c-myc protooncogene in mouse liver to activate c-myc. In the present study, DNA hypomethylation at the promoter region of the estrogen receptor α (ER α) gene was investigated by methylation-specific polymerase chain reaction (MSP) in a human breast cancer (MCF7) and in a normal (MCF10A) cell line after DBP treatment. Yeast-based estrogen receptor transcription assays showed that hER α gene expression was induced by BBP but not DBP. Moreover, MCF7 cells treated with BBP or DBP at 10^{-5} M led to the demethylation of ER α promoter-associated CpG islands. These data suggest that an altered ER mRNA expression by BBP might be related to aberrant DNA methylation in the promoter region of ER α .

The estrogen receptors (ER α and ER β) are members of the nuclear receptor superfamily of nuclear transport proteins, cell cycle regulatory components, and transcription factors (Fu et al., 2003). Most of the cellular actions of steroid hormones are mediated by their binding to nuclear receptors (NRs), which act as ligand-inducible transcription factors (Aranda & Pascual 2001). ERs activate transcription by associating with estrogen-responsive elements (ERE) located within the promoter regions of target genes (Aranda & Pascual, 2001; Robinson-Rechavi et al., 2003). Moreover, mutations in or the aberrant expressions of ERs disrupt normal development, sexual differentiation, and reproductive functions (Gao et al., 2002). In addition, the aberrant cytosine methylation of promoter regions is a potential mechanism of gene silencing or attenuation in cancer and in abnormal mammalian cells (Berger & Daxenbichler, 2002). The ER α gene contains CpG islands in its 5' upstream region (Kastner et al., 1990; Sasaki et al., 2003; Yang et al., 2003), and the abnormal methylation of such islands seems to be a frequent event in malignancies (Issa et al., 1996; Ahuja et al., 1998). DNA methylation is an epigenetic modification and is associated with transcriptional silencing in

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mammalian cells (Skiris et al., 2003). For example, the hypermethylation of promoter CpG islands was found to contribute to the loss of ER α gene function in breast cancer (Ottaviano et al., 1994; Iwase et al., 1999) and ER gene function in prostate cancer (Lau et al., 2000; Horvath et al., 2001; Nojima et al., 2001).

It is widely held that estrogens, especially estradiol-17 β (E₂), play important roles in development, growth, sexual differentiation, and reproduction in vertebrates (Kazeto et al., 2004). Considerable research efforts were expended to characterize the effects of endocrine-disrupting chemicals (EDCs) on the vertebrate endocrine system (Colborn et al., 1993; Sumpter, 1998). EDCs belong to diverse chemical classes and include pharmaceutical agents, herbicides, pesticides, plasticizers (e.g., phthalates), arylhydrocarbons (e.g., benzo[a]pyrene [BaP]), and nonionic surfactants and an associated myriad of decomposition products (Kazeto et al., 2004; DeRosa et al., 1998).

The phthalate diesters are used for a wide variety of purposes, but in terms of tonnage are predominantly used as plasticizers in polyvinyl chloride (PVC) products (Autian, 1973). Moreover, many PVC devices are produced for medical, dental, and paramedical applications, and to achieve desirable physical properties, particularly flexibility, these materials contain significant quantities of plasticizer, up to 40% by weight in finished products (Singh et al., 1972). Di(2-ethylhexyl) phthalate (DEHP) is used widely in the manufacture of PVC products and is a recognized reproductive toxicant. DEHP produces apoptosis and a reduction in spermatogenic cell counts, and may result in testicular atrophy (Kang et al., 2002; Park et al., 2002). Thus, the possible adverse effects of DEHP and other phthalate diesters are receiving considerable attention.

The perinatal administration of androgen receptor (AR) antagonists like DEHP inhibits fetal testicular testosterone production and demasculinizes males. Affected individuals display reduced anogenital distances (AGD), retained nipples, cleft phallus with hypospadias, undescended testes, a vaginal pouch, epididymal agenesis, and small or absent sex accessory glands as adults (Gray et al., 2001). In addition to DEHP, di-*n*-butyl phthalate (DBP) has been shown to display antiandrogenic activity and to induce malformations in male rats. DBP and butyl benzyl phthalate (BBP) were found to produce estrogenic effects in estrogen responsive human breast cancer cells and by recombinant yeast screening (Jobling et al., 1995; Soto et al., 1995; Coldham et al. 1997; Harris et al., 1997; Sonnenschein et al., 1995). In addition, DBP was found to produce adverse effects on reproductive-system development in rat male offspring following maternal exposure to DBP (Kim et al., 2004), and BBP has been reported to be developmentally toxic in mice and rats (Ema & Miyawaki, 2002).

The present study focused on altered methylation and ER gene expression induced by phthalates in MCF-7 and MCF10A human breast cells.

MATERIALS AND METHODS

Chemicals

Phthalate diesters, including bis(2-ethylhexyl) phthalate (DEHP, 99.0% pure), dibutyl phthalate (DBP, 98.0% pure), and butyl benzyl phthalate (BBP, 98% pure), and the phthalate monoesters, mono-2-ethylhexyl phthalate (MEHP), monobutyl phthalate (MBuP), and monobenzyl phthalate (MBeP), were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co., Inc. (Milwaukee, WI), Fluka (Steinheim, Germany) or Tokyo Kasei Kogyo (TKK, Tokyo). Procaine hydrochloride (PCA, Sigma) was dissolved in water to a final concentration of 0.27 M, sterilized by filtration, and stored at -20°C . Whenever needed, an aliquot of PCA solution was diluted to a final concentration of 10 mM (Villar-Garea et al., 2003). [^3H]-BaP (52 Ci/mmol) was purchased from Amersham Co. (Arlington Heights, IL) and BaP from Sigma. DME-F12, RPMI-1640, and Dulbecco's phosphate-buffered saline (DPBS) were also purchased from Sigma. Fetal bovine serum was purchased from InVitrogen (Carlsbad, CA,) and charcoal/dextran-treated fetal bovine solution (FBS) from HyClone (South Logan, UT).

Yeast-Based Estrogen Receptor Gene Transcription assay

Saccharomyces cerevisiae strain BJ3505 (generously donated by Dr Donald P. McDonnell, Duke University Medical Center, Durham, NC) was used for this assay. Estrogen receptor activities were determined as described previously (Gaido et al., 1997). After incubation, yeast culture samples were diluted in the appropriate selective medium to an OD_{590} , and 100 μl of this was added to each well of a 96-well microtiter plate. Samples were assayed in triplicate. Assay buffer (100 μl) (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 2 mg/ml 2-nitrophenyl- β -D-galactosidase [ONPG], 0.1% sodium dodecyl sulfate [SDS], 50 mM β -mercaptoethanol, and 200 U/ μl oxalyticase, pH 7) was then added to each well. Changes in the concentration of *ortho*-nitrophenol (the yellow product resulting from the cleavage of ONPG by β -galactosidase) were measured; β -galactosidase activities are expressed as V_{max} ($\text{mOD}_{420}/\text{min}$) divided by cell density (OD_{590}).

Methylation-Specific PCR Analysis

DNA was extracted from the cell lines using standard techniques. Bisulfite modification was carried out using the reagents included in the Chemicon CpGenome DNA modification kit (Chemicon International, Inc., Temecula, CA). Polymerase chain reaction (PCR) was performed using AmpliTaq Gold (PE Applied Biosystems, Branchburg, NJ), as described previously (Liu et al., 2003). The ER-specific primer sets (M and U primer sets; M primer, 5'-GAT ACG GTT TGT ATT TTG TTG CGC-3' and 5'-CGA ACG ATT CAA AAA CTC CAA CT-3'; U primer, 5'-GGA TAT GGT TTG TAT TTT GTT TGT-3' and 5'-ACA AAC AAT TCA AAA CTC CAA CT-3') were designed as described previously (Liu et al., 2003). Primer set U was designed to anneal to unmethylated

DNA that has undergone a chemical modification, and primer set M to anneal to methylated DNA that has undergone chemical modification. Unmethylated or methylated sequences in the ER promoter region were detected using these primer sets, respectively. PCR was performed under the following conditions: 95°C for 12 min; 40 amplification cycles (94°C for 3 min, 49°C for 45 s, 72°C for 60 s); and a final extension at 72°C for 8 min. After PCR, 10 μ l of the PCR products was mixed with 3 μ l of 6 \times loading dye and run on 2% agarose gel. Electrophoresis was carried out at 100 V at ambient temperature, and bands were visualized by ethidium bromide staining.

RT-PCR Analysis

The enzyme was activated by preheating reaction mixtures at 95°C for 6 min prior to PCR. The PCR program used was 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C with the following modifications: (a) annealing temperatures of 58 or 55°C for amplifying the ERs cDNAs; (b) 35 amplification cycles for ERs cDNAs; and (c) 26 amplification cycles for GAPDH cDNA. The primer sequences for the primer used to amplify GAPDH and the ERs were as described previously (Lau et al., 2000).

Statistical Analysis

Statistical significance was determined by using analysis of variance (ANOVA) and Scheffé's test; confidence levels were set at $p < .05$. Results are expressed as means \pm SD for at least three determinations.

RESULTS

Estrogenic Activity of Phthalates and BaP

To investigate the *in vitro* estrogenic activities of phthalates (e.g., DEHP, DBP, DEP, BBP, etc.), a yeast-based estrogen receptor gene transcription assay using *S. cerevisiae* strain BJ3505 was adopted. Chemicals were added to yeast cultures in concentrations ranging from 10^{-12} to $10^{-3}M$, yeasts were incubated with log concentrations of the indicated chemicals, and assays were performed as described in Materials and Methods. The effects of the phthalates DEHP, DBP, and BBP and their monoesters and of BaP on estrogen receptor transcription are shown in Figure 1. The synthetic estrogen diethylstilbesterol (DES), genistein, a phytoestrogen derived from soybean, and E_2 were used as positive controls. The synthetic estrogen DES was found to be as effective as estradiol in inducing β -galactosidase activity, and this was followed by coumestrol and estriol. Of the phthalates tested, BBP was found to be the only one with β -galactosidase activity. Significant β -galactosidase activity (a reporter of estrogen receptor expression) was induced by BBP at 10^{-4} and $10^{-3}M$. The remaining two phthalates (DEHP, DBP), and the phthalate monoesters (MBeP, MBuP, MEHP) did not change the β -galactosidase activity of hER α recombinant yeast strain BJ3505 at the concentrations tested. BBP was found to be

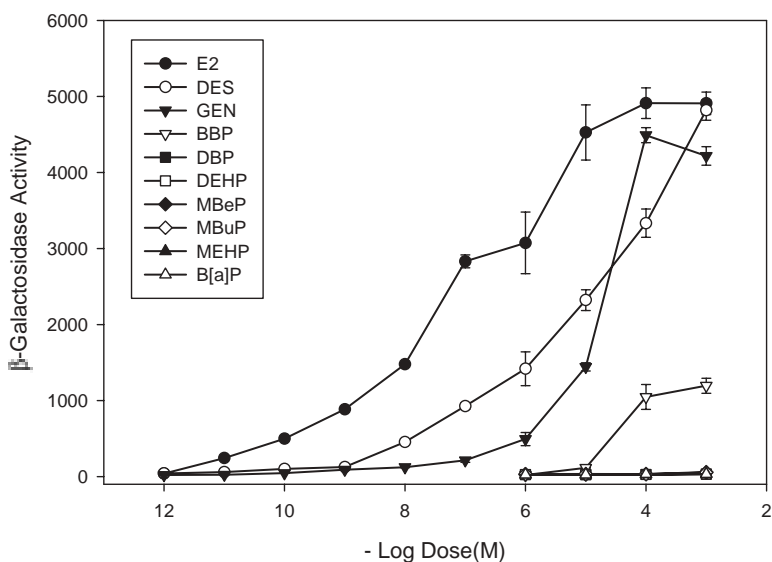


FIGURE 1. Yeast assay responses to a range of established and suspected estrogenic and anti-estrogenic phthalates. Yeasts were incubated with log concentrations of the indicated chemicals and estradiol was added to yeast cultures in concentrations ranging from 10^{-6} to 10^{-12} to $10^{-3}M$. Following overnight incubation cultures were assayed for β -galactosidase activity. Values represent the means \pm SE of three separate experiments and are presented as β -galactosidase activity (V_{max} at $O.D_{420}$) divided by the cell density (OD_{590}). E2, 17 β -estradiol; DES, diethylstilbesterol; GEN, genistein; BBP, butyl benzyl phthalate; DBP, dibenzyl phthalate; DEHP, di(2-ethylhexyl)phthalate; MBeP, monobenzyl phthalate; MBuP, monobutyl phthalate; MEHP, monoethyl hexyl phthalate; BaP, benzo[a]pyrene.

weakly estrogenic by the yeast-based estrogenicity assay in BJ3505 strain, whereas the BBP metabolites (MBeP, MBuP) all lacked such activity.

Methylation Status of the ER α Promoter in the Human Breast Cell Lines

Methylation-specific PCR (MSP) analysis showed methylated bands of ER α promoter in all phthalate-treated cells, MCF-7 (cancer cell line) and MCF10A (normal cell line), whereas unmethylated bands were observed only in MCF-7 cells treated with BBP and DBP (Figure 2). In the DEHP- and BaP-treated cells, no unmethylated bands were observed (data not shown). Procaine (PCA), a demethylating agent, was used as a positive control. PCA produces a growth-inhibitory effect on human breast cancer cells, which is associated with mitotic arrest due to its direct binding with CpG-rich DNA (Villar-Garea et al., 2003).

Induction of ERs Genes by Phthalates in MCF-7 Cells

Treating MCF-7 cells with PCA, BBP, or DBP at $10^{-5}M$ led to the demethylation of the ER α promoter-associated CpG island and restored the expression of the ER α transcript (Figures 2 and 3). The induction of demethylating events in the CpG island of the ER α gene was associated with the expression of ER α

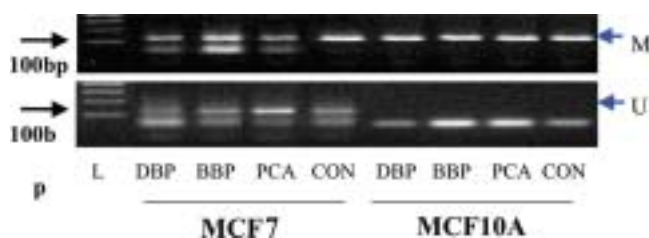


FIGURE 2. Methylation status of the ER α promoter in the MCF-7 breast cancer cell line and in the MCF10A normal cell line after phthalate treatment. Cytosine residues in CpG sites were converted to thymine after bisulfite treatment and PCR. M, methylated bands; U, unmethylated bands. L, 100-bp DNA ladder; DBP, 10^{-5} M dibenzyl phthalate; BBP, 10^{-5} M butyl benzyl phthalate; PCA, 10^{-5} M procaine; CON, DMSO.

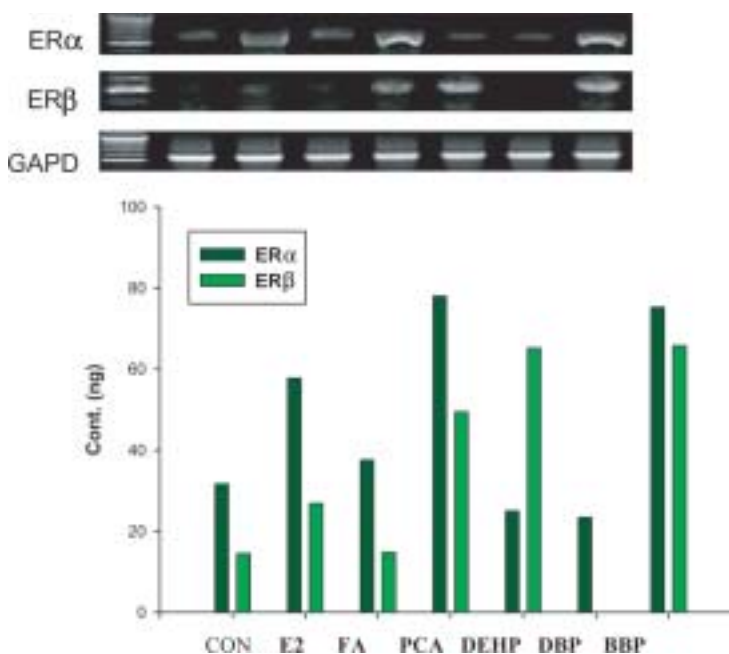


FIGURE 3. Reactivations of the mRNA expressions of ER α and ER β in MCF-7 cells by phthalates. Total RNAs were extracted and reverse transcribed. The resulting cDNAs were subjected to PCR analyses under optimized conditions. Amplified products were run in 2% agarose gel containing ethidium bromide. CON, DMSO; E₂, 10^{-8} M 17 β -estradiol; FA, 10^{-5} M folic acid; PCA, 10^{-5} M procaine; 10^{-5} M DEHP, DBP, and BBP.

mRNA, and the DNA was unmethylated in untreated MCF-7 cells (Figure 3). ER α was expressed in MCF-7 cells treated with BBP, and ER β was expressed in DEHP- and BBP-treated cells (Figure 3). Folic acid (FA), a methyl donor, did not alter ER α expression levels compared to control dimethyl sulfoxide (CON, [DMSO]).

DISCUSSION

The estrogenic effects of endocrine-disrupting chemicals (EDCs) are a major concern due to developmental toxicity, carcinogenicity, mutagenicity, and immunotoxicity (DeRosa et al., 1998; Choi et al., 2004). Therefore, the estrogenic activities of EDCs such as plasticizers (e.g., DEHP, DEP, DBP, etc.) and PAH (BaP) were tested using yeast-based estrogen receptor transcription assays using the hER α recombinant yeast strain BJ3505. In our study, a concentration-related increase in the expression of the estrogen receptor transcript was observed in cells treated with BBP, which was suggestive of estrogenicity. BBP was found to be weakly estrogenic by MCF-7 cell proliferation assays, whereas BBP metabolites lacked such activity (Picard et al., 2001). These findings agree with these reports, since BBP was found to be weakly estrogenic, whereas MBeP and MBuP (possible major metabolites of BBP) were not, in our yeast-based estrogen receptor transcription assay.

Recent studies aroused increasing interest in the role of DNA hypermethylation in tumorigenesis via its ability to alter tumor suppressor gene expressions. In general terms, the hypermethylation of promoter CpG islands contributes to the loss of gene function of several tumor-related genes, including ERs (Baylin & Herman, 2000; Yan et al., 2001). Sex hormone targeted nuclear receptors (NRs) genes, such as ER, AR, and PR, contain CpG islands in the 5' upstream region (Kastner et al., 1990; Sasaki et al., 2003; Yang et al., 2003). Ottaviano et al. (1994) reported that the CpG island located in the promoter region and the first exon of ER α were methylated in ER-negative breast cancer cell lines, but found no methylation in ER-positive breast cancer cell lines or in normal breast tissues. Indications of an association between promoter methylation and a loss of ER α expression were further provided by the finding that treating various cell lines with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) restored the ER α expression and transcription of estrogen-responsive genes (Ferguson et al., 1995; Lau et al., 2000; Liu et al., 2003).

In the present study, the methylation status of ER α gene was investigated in human breast cell lines. Data showed that the methylation status of the ER α gene promoter was altered by BBP and DBP in the MCF-7 human breast cancer cell line. Treatment of MCF-7 cells with procaine (PCA), BBP, or DBP led to the demethylation of the ER α promoter-associated CpG island and restored ER α transcript expression. These results suggest that BBP and DBP induced-DNA hypomethylation or -demethylation may modulate the expression of a CpG-island-associated gene (ER α).

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