Phthalates Stimulate the Epithelial to Mesenchymal Transition Through an HDAC6-Dependent Mechanism in Human Breast Epithelial Stem Cells

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Phthalates are environmental hormone-like molecules that are associated with breast cancer risk and are involved in metastasis, a process that requires the epithelial-mesenchymal transition (EMT). However, few studies have addressed the potential effects of phthalates on stem cells. Here we tested the hypothesis that phthalates such as butyl benzyl phthalate and di-α-butyl phthalate induce EMT in R2d cells, a stem cell–derived human breast epithelial cell line that is responsive to estradiol for tumor development. We observed that phthalates induced EMT as evidenced by morphological changes concomitant with increased expression of mesenchymal markers and decreased expression of epithelial markers. Molecular mechanism studies revealed that histone deacetylase 6 (HDAC6) is required for phthalate-induced cell migration and invasion during EMT in vitro and metastasis into the lungs of nude mice. We also constructed a series of mutant HDAC6 promoter fragments and found that the transcription factor AP-2a plays a novel role in regulating the HDAC6 promoter. Furthermore, phthalates stimulated estrogen receptors and triggered the downstream EGFR–PKA signaling cascade, leading to increased expression of AP-2a in the nucleus. We also observed that phthalates increased expression of the PP1/HDAC6 complex and caused Akt activation and GSK3β inactivation, leading to transcriptional activation of vimentin through the β-catenin–TCF-4/LEF1 pathway. Understanding the signaling cascades of phthalates that activate EMT through HDAC6 in breast epithelial stem cells provides the identification of novel therapeutic target for human breast cancer.

Key Words: environmental hormone; phthalates; EMT; HDAC6; AP-2a.

Phthalates are mainly used as plasticizers and are commonly found in a variety of household, food, medical, and cosmetic products. Some phthalates, such as butyl benzyl phthalate (BBP) and di-α-butyl phthalate (DBP), have weak estrogenic activity (Jobling et al., 1995; Moore, 2000). Therefore, phthalates are considered endocrine disruptors. Earlier research focused on phthalates’ endocrine disrupting effects in men. More recently, the endocrine disrupting effects of these compounds in women have been documented in regard to breast cancer risk. Epidemiological evidence suggests that breast cancer risk increases following exposure to diethyl phthalate in the environment (Lopez-Carrillo et al., 2010). Furthermore, phthalates reportedly block cell-to-cell communication (Malcolm and Mills, 1989), a property of tumor-promoting chemicals, and promote tumor growth (Ward et al., 1983, 1984). Phthalates also affect cell proliferation and inhibition of tamoxifen-induced apoptosis in estrogen receptor (ER)–positive MCF-7 cells but not in ER– MDA-MB-231 cells (Kim et al., 2004). The stimulation of proliferation of MCF-7 cells by BBP and DBP can be completely suppressed by the ER antagonist, ICI182780 (Okubo et al., 2003). In addition, phthalates promote invasion and metastasis of SK-N-SH human neuroblastoma cells by inducing cell motility that is mediated by matrix metalloproteinase-2 and -9 expression through the PI3K/Akt pathway (Zhu et al., 2010). Cell motility is an important feature in tumor progression following the epithelial-mesenchymal transition (EMT) (Thiery, 2002; Thompson et al., 2005; Xue et al., 2003), which may be regulated by estrogen (Ding et al., 2006) and transforming growth factor-beta (TGF-β) (Valcourt et al., 2005).

The cell line M13SV1R2 was isolated from a normal human breast epithelial cell type that has stem cell characteristics (Kang et al., 1998). This cell line was created by immortalization of the parent line via expression of SV40 large T-antigen and subsequent transformation by X-irradiation. When
cultured in growth factor/hormone-deprived medium, the cell line (referred to as R2d cells) is not tumorigenic. However, these R2d cells are responsive to estrogen, which promotes cell growth, EMT, and tumor development (Wang et al., 2010a).

In our current study, we stimulated R2d cells with phthalates, used an Illumina cDNA microarray to carry out a gene ontology analysis, and assessed the data with Ingenuity Pathways Analysis Software. The results showed that one of the major pathways affected by phthalates governs developmentally regulated morphological changes (Supplementary fig. 1). Based on these findings, we examined molecular mechanisms of neoplastic transformation induced by phthalates. Particularly, we focused on the role of histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, which has been shown to regulate the cytoskeleton (Hubbert et al., 2002; Valenzuela-Fernandez et al., 2008) and may play an important role in EMT and cell motility (Fernandez and Russo, 2010). Furthermore, HDAC6 is required for invadopodia formation, breast cancer metastasis (Rey et al., 2010), and efficient oncogenic transformation (Lee et al., 2008b). Our previous study also showed that HDAC6 has an important role in promoting stem cell–mediated breast tumorigenesis (Wang et al., 2010b) that is induced by 1 μmol/l phthalates in ER– breast cancer cells (Hsieh et al., 2011). Based on these previous observations, we examined the molecular mechanism of phthalate-induced EMT and tumor development, with particular attention given to the role of HDAC6 in R2d cells.

MATERIALS AND METHODS

Cell lines and chemicals. The cancer stem cell line R2d was a kind gift from Prof. C.-C. Chang (Michigan State University, East Lansing, MI). The cells were maintained in basic MSU-1 (Kao et al., 1995) medium with 10% fetal bovine serum (Sigma, St Louis, MO) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). R2d and MCF-7 (ATCC) cells were cultured in a humidified atmosphere with 5% CO2 at 37°C. The drugs and inhibitors, including BBP (1 μmol/l)(Chen et al., 2011), DBP (1 μmol/l), trichostatin A (TSA, 2 μmol/l), IC1182780 (1 μmol/l), AG1478 (10 μmol/l), and H89 (1 μmol/l), were purchased from Calbiochem (San Diego, CA).

Immunoblotting. To determine the expression of EMT marker proteins, R2d cells were subcultured in 6-well plates (5 × 10⁵ cells per well). After 24 h, the cells were cultured in serum-free MSU-1 medium for 24 h, followed by treatment with 1 μmol/l BBP or DBP for various periods. Protein lysates were obtained using the M-PER mammalian protein extraction reagent (Thermo Scientific, Franklin, MA) and separated on SDS-PAGE gels (37% acrylamide). Proteins were blotted onto a polyvinylidene fluoride (Millipore Corp., Bedford, MA) and separated on SDS-PAGE gels (37% acrylamide). Protein lysates were separated on 2% agarose gels and stained with ethidium bromide. QPCR expression at the mRNA level, reverse transcription-PCR (RT-PCR) and quantitative PCR assay (QPCR). To confirm gene expression at the mRNA level, reverse transcription-PCR (RT-PCR) and quantitative PCR (QPCR) assays were carried out. R2d cells (5 × 10⁴) were refreshed and cultured in a 6-well plate. After 24 h, the cells were cultured in serum-free MSU-1 medium for 24 h, followed by treatment with 1 μmol/l BBP or DBP for various periods. Total RNA was extracted from R2d pellets using 1 ml TRIzol (Invitrogen). RNA (2 μg) was transcribed into cDNA with a Reverse Transcription System Kit (Promega, Madison, WI), and cDNAs were amplified with gene-specific primers in a PCR machine (Eppendorf). The primers (Gene messenger Scientific, Tainan, Taiwan) used in the experiment were: human HDAC6: forward, 5'-CACAACACGAGCCACGGCAAGAG-3' and reverse, 5'-ATCCATCCCTTGGTACGCGCAG-3'; vimentin: forward, 5'-AGCAAGGGAGTCTTTCAGGCGCCG-3' and reverse, 5'-GGAGAGGATTGTCTTGCAGG-3'; cytokeratin 7: forward, 5'-CCGAGCTCCCAGAATGACAA-3' and reverse, 5'-CCCAAGATGGCTCAGGTACCA-3'; and β-actin (loading control): forward, 5'-ATGATATCGCCGCGCTCGTCGTC-3' and reverse, 5'-GAACCACAACGTGTTGGAGAG-3'. The amplified products were separated on 2% agarose gels and stained with ethidium bromide. QPCR Analysis Software. The results showed that one of the major products amplified with gene-specific primers in a PCR machine (Eppendorf). The images were captured by a light microscope, and the cells were counted in three different fields.

Reverse transcription-PCR and quantitative PCR assay. To confirm gene expression at the mRNA level, reverse transcription-PCR (RT-PCR) and quantitative PCR (QPCR) assays were carried out. R2d cells (5 × 10⁴) were refreshed and cultured in a 6-well plate. After 24 h, the cells were cultured in serum-free MSU-1 medium for 24 h, followed by treatment with 1 μmol/l BBP or DBP for various periods. Total RNA was extracted from R2d pellets using 1 ml TRIzol (Invitrogen). RNA (2 μg) was transcribed into cDNA with a Reverse Transcription System Kit (Promega, Madison, WI), and cDNAs were amplified with gene-specific primers in a PCR machine (Eppendorf). The primers (Gene messenger Scientific, Tainan, Taiwan) used in the experiment were: human HDAC6: forward, 5'-CACAACACGAGCCACGGCAAGAG-3' and reverse, 5'-ATCCATCCCTTGGTACGCGCAG-3'; vimentin: forward, 5'-AGCAAGGGAGTCTTTCAGGCGCCG-3' and reverse, 5'-GGAGAGGATTGTCTTGCAGG-3'; cytokeratin 7: forward, 5'-CCGAGCTCCCAGAATGACAA-3' and reverse, 5'-CCCAAGATGGCTCAGGTACCA-3'; and β-actin (loading control): forward, 5'-ATGATATCGCCGCGCTCGTCGTC-3' and reverse, 5'-GAACCACAACGTGTTGGAGAG-3'. The amplified products were separated on 2% agarose gels and stained with ethidium bromide. QPCR Analysis Software. The results showed that one of the major products amplified with gene-specific primers in a PCR machine (Eppendorf). The images were captured by a light microscope, and the cells were counted in three different fields.

Flow cytometry. After culturing the R2d and MCF-7 cells in serum-free medium for 24 h, cells were treated with 1 μmol/l of each phthalate. After 24 h, cells in culture dishes were washed with ice-cold PBS, trypsinized, fixed with 70% ethanol, and transferred to a centrifuge tube. These cells were then incubated at 37°C for 2 h with different labeled antibodies and detected based on their protein expression using a flow cytometer (Beckman Coulter, Miami, FL). The following antibodies were used: anti-CD90 (1:1000; Epitomics), anti-vimentin (1:100; Santa Cruz Biotechnology), anti-cytokeratin 7 (1:1000; Dako, Hamburg, Germany), anti-E-catenin (1:1000; BioLegend, San Diego, CA), anti-HDAC6 (1:100; Santa Cruz Biotechnology), and anti-acetylated tubulin (1:5000; Sigma).

Immunofluorescence. R2d cells (1 × 10⁴ per well) were seeded in 6-well plates that were covered with glass coverslips. After 24 h, the cells were cultured in serum-free MSU-1 medium for 24 h and treated with various chemicals including phthalate (1 μmol/l), AG1478 (10 μmol/l), and H89 (1 μmol/l). Cells were rinsed with ice-cold PBS, fixed with 3.7% formaldehyde (Sigma) in PBS for 30 min, permeabilized with 0.2% Triton X-100 (Calbiochem) for 20 min, and blocked in 3% bovine serum albumin in PBS. The following primary antibodies were used for immunofluorescence: anti-β-actin (1:5000; Sigma), anti-HDAC6, and anti-AP-2α (1:100; Santa Cruz Biotechnology). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (10 μg/ml, Sigma), and glass coverslips were carefully placed onto microscope slides and mounted. Microscopic images were obtained using a microscope (IX-71, Olympus, Tokyo, Japan).

Wound-healing and invasion assays. For the wound-healing assay, R2d cells (5 × 10⁴ per well) were refreshed and cultured in a 6-well plate. At 90% confluence, the cells were cultured in serum-free MSU-1 medium for 24 h. The cells were scratched with a micropipette tip (to create an artificial wound/gap) and treated with 1 μmol/l phthalates for 24 h. To quantify relative cell migration distances, we measured the width of the remaining gap (surface not covered by cells) using an Olympus IX-71 microscope in three different fields. We compared the wound distance between the phthalate-treated and control groups. The invasiveness of R2d cells was examined and evaluated with a Cell Invasion Assay kit (Chemicon, Temecula, CA). The images were captured by a light microscope, and the cells were counted in three different fields.

Immunoprecipitation. Protein lysates from treated R2d cells were obtained using the M-PER mammalian protein extraction reagent (Thermo Scientific). After centrifugation of cell lysates at 12,000 rpm for 10 min (EPPENDORF, 5810 R, Hamburg, Germany), protein-G beads (Roche, Indianapolis, IN) were mixed with the immunoprecipitates and incubated at 4°C for 2 h with rocking to eliminate nonspecific binding. The beads were removed, and each supernatant received fresh beads that had been prebound with anti-PP1 (protein phosphatase-1) (Santa Cruz Biotechnology) to detect the presence of PP1/Akt or PP1/HDAC6 complexes; the entire mixture was then incubated at 4°C for 24 h with rocking. After centrifugation to separate the beads, the antibody-protein complexes were eluted from the beads with washing buffer, and the supernatant was used for immunoblotting for HDAC6, Akt, and PP1.
experiments were carried out using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and the results were analyzed using an ABI 7500 Real-Time PCR System (Applied Biosystems).

siRNA transfection. To investigate whether HDAC6 and Ap-2α are required to promote EMT in cells treated with BBP and DBP, specific short interfering RNAs (siRNAs) were used to silence the expression of HDAC6 and Ap-2α. First, R2d cells were grown in OPTI-MEM medium ( Gibco, Grand Island, NY). After reaching 80% confluence, cells were transfected using the LT1 transfection reagent (Merus, Madison, WI) according to the manufacturer’s protocol. The following siRNAs were used: control siRNA (sense: 5′-GAUAUAACGGCCAGUCAAGC-3′, antisense: 5′-UCUGAUAGCAGUAUAUACCC-3′; Sigma). HDAC6 siRNA-1 (GenBank NM_006044; sense: 5′-GCAUUAACCUUUAUACCC-3′, antisense: 5′-UCUAGGAAUAAGAGAUCG-3′; Sigma), HDAC6 siRNA-2 (GenBank SASI_0048982; sense: 5′-CACUCAUGACCAGC-3′, antisense: 5′-UCUUUAACUUAGUUGAC-3′; Sigma), and Ap-2α (GenBank no. SASH2_0080715; sense: 5′-GAUUAACCAUGC-3′, antisense: 5′-UCUUAAUACUAAGGUG-3′; Sigma) at a final concentration of 5 nM. Following transfection, data were obtained using Western blotting, immunofluorescence, the motility assay, and chromatin immunoprecipitation (ChIP) experiments.

ChIP assay. Following phthalate treatment, we used a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) to determine whether the transcription factor Ap-2α binds to the HDAC6 promoter and whether TCF-4/LEF1 binds to HDAC6 or vimentin promoter region. The HDAC6 primer (Genemessner Scientific) sequences were: forward, 5′-GGCCCAATGGAAGAA-3′ and reverse, 5′-CCCTCAACCAGC TTCAC-3′. The vimentin promoter region sequences were: forward, 5′-GGCTGCGGCAGGGAACCTG-3′ and reverse, 5′-GGGCGGGAGAGGAAACG-3′. We used PCR to amplify the transcription factor–binding sequence. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

Molecular constructs and dual luciferase reporter assays. To explore potential regulatory elements in the 5′-promoter region of human HDAC6, a series of promoter-derived fragments were constructed and cloned into vector pGL3 (Promega). The human HDAC6 promoter sequence was amplified from genomic DNA with PCR using primers that contained an MluI (forward primer) or NheI site (reverse primer) at their 5′ ends: forward primers (Genemessner Scientific) 5′-ACGACCGCTGTCGCTTTCTTTCG-3′; reverse primers 5′-ACGACCGCTGTCGCTTTCTTTCG-3′; and reverse primers 5′-ACGACCGCTGTCGCTTTCTTTCG-3′. PCR products were obtained using the pGL3-basic basic expression vector (Promega). We further examined whether Ap-2α could bind the promoter sequence directly, and mutant fragments were constructed and cloned for this purpose. Mutant HDAC6 promoter plasmid was created using site-directed mutagenesis with the forward primer 5′-ACGACCGCTGTCGCTTTCTTTCG-3′ and reverse primer 5′-ACGACCGCTGTCGCTTTCTTTCG-3′. For the dual luciferase reporter assays (Promega), R2d cells were cotransfected with HDAC6 promoter plasmids and the Renilla luciferase expression vector using the LT1 transfection reagent for 24 h. After 1 μmol/l phthalate treatment for 6 h, dual luciferase activities were quantitated with the ELISA reader, and Renilla luciferase activity was normalized to firefly luciferase activity. Data are the mean ± SEM of three experiments.

Subcellular fractionation. To assess protein translocation, the nuclear and cytoplasmic fractions of R2d cells were isolated using the Compartmental Protein Extraction kit CMNCs (Biochain, Hayward, CA) according to the manufacturer’s protocol. Protein samples (50 μg) from the different fractions were subjected to immunoblotting using anti-Ap-2α and anti-β-catenin.

Furthermore, the anti-alpha-tubulin (cytoplasmic marker) and anti-histone 3 (nuclear marker) were used to check the purity of the fractions.

Animal studies. R2d cells were infected with viruses carrying plK01.1-YFP or transfected with pLKO.1-GFP-HDAC6 short hairpin RNA (shRNA) plasmids (both obtained from the National RNAi Core Facility, Academia Sinica, Taiwan) according to the RNAi Core Facility’s protocol. The shRNAs for yellow fluorescent protein (YFP) and green fluorescent protein (GFP)–HDAC6 were stably expressed in R2d-YFP and R2d-GFP-HDAC6 shRNA cell lines, respectively. All animal studies were performed according to a protocol approved by the Kaohsiung Medical University Hospital Institutional Animal Care and Use Committee. Female nude mice (BALB/cAnN.Cg-Foxn1nu/Crl-Narl, 4 to 6 weeks old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). R2d-YFP or R2d-GFP-HDAC6 shRNA cells (2 × 106) were intravenously injected into nude mice, which were then randomly divided into three groups (n = 6 each group): saline control, BBP, and DBP. A previous report showed that BBP at doses up to 800 mg/kg, when injected into the peritoneum, showed no significant toxicity in mice after 24 weeks (Singletary et al., 1997). Therefore, treatment with 800 mg/kg for 8 weeks was selected for this experiment. Mice were imaged 8 weeks after the cells were injected using an Ultra Sensitive Molecular Imaging System (Berthold Technologies, Bad Wildbad, Germany) to detect metastases and the location of tumor growth in vivo.

RESULTS

Phthalates Induce EMT, Cell Migration, and Invasion

To evaluate the effect of phthalates on EMT, the level of protein markers for mesenchymal and epithelial cells was analyzed by Western blotting. R2d cells were treated with phthalates for 24, 48, or 72 h; subsequent analysis of the expression of EMT markers revealed a significant increase in the level of vimentin and reduced level of cytokeratin 7 (Fig. 1A). EMT is a complex cellular process that involves coordinated changes at multiple levels and locations within cells. Therefore, we also used other markers of EMT, including the mesenchymal markers vimentin and CD90 and epithelial markers cytokeratin 7 and E-cadherin. As shown in Figure 1B, BBP or DBP treatment (1 μmol/l) for 24 h enhanced expression of the mesenchymal markers vimentin and CD90 and decreased the epithelial markers cytokeratin 7 and E-cadherin. Treatment with BBP or DBP changed the typical epithelial cell morphology and contiguous colony appearance of R2d cells into more spindle-like cells and scattered colonies, resembling mesenchymal cells. These morphological changes were observed after 72 h of treatment with phthalates (Fig. 1C). To further test whether phthalate-treated cells changed their migration and invasive ability associated with EMT, wound healing and invasion assays were performed. We found that phthalates significantly enhanced the migration (Fig. 1D) and invasion ability of R2d cells (Fig. 1E) compared with the untreated control. Thus, phthalates are capable of inducing EMT and the invasive ability of breast epithelial stem cells.

HDAC6 Is Required for Phthalate-Mediated Induction of EMT

To determine whether HDAC6 protein expression is required for EMT induction by phthalates, we first analyzed the protein level of HDAC6 and the amount of acetylated tubulin by flow
cytometry of R2d and MCF-7 cells treated with phthalates. BBP or DBP treatment (1 μmol/l) for 24 h significantly increased the expression of HDAC6 and concomitantly reduced the amount of acetylated tubulin (Fig. 2A). Phthalates had the
FIG. 2. HDAC6 is required for phthalate-mediated EMT and cell migration and invasion. (A) Detection of HDAC6 and acetylated tubulin with flow cytometry in R2d, MCF-7 cells treated with or without 1 μmol/l phthalates. (B) HDAC6 and acetylated tubulin were analyzed in cells transfected with HDAC6 siRNA-1 (5 ng/μl) and HDAC6 siRNA-2 (5 ng/μl) compared with control siRNA (5 ng/μl) cells. Total protein was extracted and subjected by Western blotting. (C) R2d cells were pretreated with 2 μmol/l TSA, an HDAC inhibitor, for 30 min or transfected with HDAC6 siRNA-1/-2 (5 ng/μl), followed by exposure to 1 μmol/l of each phthalate. Protein levels of HDAC6, acetylated tubulin, vimentin, and cytokeratin 7 were examined with immunoblotting, and RNA levels of HDAC6, vimentin, and cytokeratin 7 were determined with RT-PCR 24 h after exposure. (D) R2d cells were transfected with control siRNA (5 ng/μl), HDAC6 siRNA-1 (5 ng/μl), or HDAC6 siRNA-2 (5 ng/μl) after treatment with phthalates for 72 h, and the morphology was observed using Alexa 488–conjugated actin. (E and F) R2d cells as described in (D). The migration and invasion ability was examined 24 h after exposure to 1 μmol/l phthalates using migration and invasion assays. Data are the mean ± SEM of three experiments. *p < 0.05 compared with the untreated control (one-tailed Student’s t-test). C, control.
FIG. 3. The AP-2a binding site is located between –818 and –589 of the human HDAC6 promoter region. (A) HDAC6 promoter activity was analyzed in R2d cells treated with phthalates. Various HDAC6 reporter constructs were transfected into R2d cells. At 24-h post-transfection, R2d cells were stimulated with 1 μmol/l phthalates (BBP and DBP), and lysates were collected to analyze luciferase activity. Luciferase activities of phthalate-treated cells were compared with activity of untreated cells. (B) Binding of the transcription factor AP-2a to the HDAC6 promoter as analyzed by ChIP after treatment with or without 1 μmol/l phthalates (BBP and DBP). At 48 h after treatment with phthalates, AP-2a siRNA (5 ng/μl) was transfected into R2d cells for 24 h. ChIP was conducted with anti-AP-2a and an unrelated control antibody (IgG); IP, immunoprecipitation. DNA was amplified with primers specific for HDAC6. Genomic DNA was used to normalize the DNA used for IP (input). (C) R2d cells were transfected with AP-2a-specific siRNA followed by treatment with 1 μmol/l phthalates (BBP and DBP). Immunoblots were performed to detect AP-2a, HDAC6, and acetylated tubulin. β-actin was used as a loading control. C, control. HDAC6 protein levels were quantified by ImageJ software (U.S. National Institutes of Health, Bethesda, MD).
same effect on both the R2d and MCF-7 cells. To investigate whether HDAC6 is required for the EMT promoting effects of BBP and DBP, HDAC6 was silenced by specific siRNAs (Fig. 2B). These data confirmed that siRNA decreased the level of HDAC6 protein and consequently increased the level of acetylated tubulin. To confirm that HDAC6 mediates the effect of phthalates on EMT, cells were transfected with a construct encoding siRNA targeting HDAC6 mRNA or pretreated with the HDAC inhibitor TSA for 24 h, and the expression of the EMT markers vimentin and cytokeratin 7 was examined with Western blotting and RT-PCR (Fig. 2C). The results showed that inhibiting HDAC6 expression prevented the effects of the phthalates. Similarly, HDAC6 siRNA-1 and 2 treatment prevented the EMT-like morphological changes (Fig. 2D) and blocked the cell migration (Fig. 2E) and invasion (Fig. 2F) induced by BBP or DBP. Thus, HDAC6 expression is essential for the phenotypic expression of EMT, cell migration, and invasion that are induced by phthalates.

The Transcription Factor AP-2a Binds to the HDAC6 Promoter Following Phthalate Exposure

We analyzed whether the HDAC6 promoter was activated by the transcription factor following phthalate treatment. First, we used an NCBI database sequence to design promoter fragments with 5’ deletions (-1037, -818, -589, -372, -252, and -147) to 3’ regions of the promoter and transfected them into R2d cells to test reporter expression in response to phthalate treatment. The luciferase response increased significantly with fragments -1037/-44 and -818/-44 following phthalate treatment; the smaller constructs (-589, -372, -252, and -147/-44), however, did not produce such a response. Thus, the transcription factor–binding site in the promoter region was localized between -818 and -589. We used MetaCore programs to deduce the putative transcription factor–binding sites and constructed fragments from -818 to -589. Within this sequence, we identified the binding motif for AP-2a (GCCNNNGGC) in the regulatory region of the promoter (Imagawa et al., 1987). We used site-directed mutagenesis to alter the sequence (from GCCAAGGGC to GTCAAGGTGT) to see whether the mutated sequence resulted in dramatic reduction of luciferase response following phthalate treatment. The mutated motif negated the phthalate effect on HDAC6 reporter expression (Fig. 3A). The phthalate-dependent response of reporter gene activity differed for the various constructs.

Using ChIP and Western blotting, we further examined whether AP-2a binding to the HDAC6 promoter occurred upstream of HDAC6 expression following phthalate treatment. The ChIP assay (Fig. 3B) showed that phthalate treatment increased AP-2a binding to the promoter; this binding was blocked by AP-2a siRNA. Western blotting (Fig. 3C) showed that AP-2a siRNA inhibited HDAC6 expression and enhanced tubulin acetylation after phthalate treatment. Thus, AP-2a is essential for mediating HDAC6 protein expression that is induced by treatment with phthalates.

Activation of the ER/Epidermal Growth Factor Receptor/Protein Kinase A Pathway Is Required for Localization of AP-2a in Nuclei Following Phthalate Treatment

Our current data showed that phthalates activate protein kinase A (PKA) and therefore we tested whether ER and epidermal factor receptor (EGFR) are upstream regulators of PKA in phthalate-treated R2d cells. According to previous studies, PKA may act upstream of AP-2a activation (Yang et al., 2008), and ERs regulate PKA via EGFR and Src (Laag et al., 2006; Thoennissen et al., 2010). We determined whether phosphorylation of EGFR at Y1173 and phosphorylation of Src at Y416 were activated by phthalates. We observed that BBP and DBP treatment enhanced pY1173 in EGFR and pY416 in Src in R2d cells in a time-dependent manner (Fig. 4A). In addition, we examined whether EGFR and Src were downstream of ER. We observed that the ER inhibitor IC1182780 decreased the phosphorylation of EGFR and Src during treatment of R2d cells with BBP or DBP, without affecting the total amounts of EGFR and Src protein (Fig. 4B). We further tested whether the EGFR inhibitor AG1478 and the PKA inhibitor H89 blocked the entry of AP-2a into the nucleus following treatment with phthalates. Western blotting of subcellular fractions showed that AG1478 and H89 inhibited AP-2a translocation into the nucleus after phthalate treatment (Fig. 4C). As expected, alpha-tubulin and histone 3 were found predominantly in the cytosol and nuclear fractions, respectively (Fig. 4C). Moreover, phthalate treatment resulted in a marked increase in AP-2a and HDAC6 immunofluorescence (Fig. 4D). Phthalate-induced AP-2a translocation to the nucleus was blocked by inhibitors of EGFR and PKA. Thus, ER, EGFR, and PKA play important roles in the phthalate-induced effects that are mediated by HDAC6 through an epigenetic mechanism.

The Akt/PP1/β-Catenin Pathway Is Involved in HDAC6-Mediated EMT Following Phthalate Treatment

The Akt/β-catenin pathway is an essential link for signal transduction leading to EMT induction (Li and Zhou, 2011). Expression of the mesenchymal marker, vimentin, has been suggested to involve the β-catenin pathway, in which phosphorylation of Akt leads to the release of active β-catenin into the nucleus, which in turn mediates TCF-4/LEF1 binding to the vimentin promoter (Gilles et al., 2003; Lee and Han, 2009; Lee et al., 2008a; Medici et al., 2006; Nawshad et al., 2007). A previous study also showed that TSA abrogates Akt phosphorylation by disrupting PP1/HDAC complexes and promoting association between PP1 and Akt, leading to Akt dephosphorylation (Lin et al., 2006). PP1 plays an important role in mediating dephosphorylation of serine and threonine residues. Earlier reports showed that PP1 increases apoptosis through Akt dephosphorylation in prostate cancer (Garcia et al., 2003; Li et al., 2003). Thus, we investigated the relationship between HDAC6 function and the activity of the PP1/Akt complex.
after treatment with phthalates. Western blotting showed that HDAC6 siRNA-1 inhibited the expression of phosphorylated Akt (p-Akt) and p-GSK3β when R2d cells were stimulated with phthalates (Fig. 5A). Thus, after phthalate treatment, HDAC6 increased the phosphorylation of Akt and GSK3β, activating Akt and inactivating GSK3β. In contrast, the PP1/HDAC complex caused Akt phosphorylation and activation of the β-catenin signaling pathway. To test the involvement of Akt phosphorylation induced by phthalates, we used immunoprecipitation to analyze the PP1/Akt and PP1/HDAC6 complexes. We observed that TSA and HDAC6 siRNA-1 blocked PP1 binding to HDAC6 when cells were treated with phthalates (Fig. 5B). In addition, we used a compartmental protein extraction kit and ChIP to examine whether phthalates induced the release of activated β-catenin into the nucleus and affected TCF-4/LEF1 binding to the vimentin promoter. Phthalates led to a significant increase of β-catenin in nuclei (Fig. 5C) and activated the binding of TCF-4/LEF1 to the vimentin promoter (Fig. 5D). In contrast, treatment with TSA or HDAC6 siRNA-1 prevented the localization of β-catenin to the nucleus and blocked TCF-4/LEF1–mediated transcriptional activation. Taken together, these data suggest that BBP and DBP regulate vimentin expression by disrupting the interaction between HDAC6 and the Akt/PP1 complex, which results in nuclear localization of β-catenin and activation of the transcription factor TCF-4/LEF1.

**HDAC6 Is Involved in Phthalate-Induced Tumorigenesis and Metastasis of R2d Cells in Nude Mice**

Previous studies showed that tumorigenic cells with mesenchymal phenotypes have strong metastatic potential (Hayashida et al., 2011). Therefore, we investigated the effect of phthalates on metastasis in vivo. We used lentivirus infection to label two groups of R2d cells with YFP and GFP (Fig. 6A). The GFP-encoding cells were transfected with a shRNA targeting HDAC6 mRNA to knock down the HDAC6 level. The difference in HDAC6 expression in these two groups of cells was confirmed by analyzing mRNA expression with QPCR (Fig. 6B) and protein expression with Western blotting (Fig. 6C). HDAC6 expression was markedly decreased in the R2d-GFP HDAC6 shRNA cells, and acetylated tubulin was increased in the R2d-GFP HDAC6 shRNA cells compared with R2d or R2d-YFP cells. We compared the metastatic ability of these two groups of cells after treatment with phthalates. For these experiments, 2 × 10^5 R2d-YFP or R2d-GFP HDAC6 shRNA cells were intravenously injected into nude mice, which were
subsequently treated with ip injections of phthalates (800 mg/kg/day) or vehicle control. Whole-body bioluminescence detection was used to detect metastasis after 8 weeks. As shown in Figure 6D, BBP and DBP promoted lung colonization in mice injected with R2d-YFP cells (BBP: 5/6 mice; DBP: 6/6 mice), whereas no colonization was observed in mice injected with R2d-GFP HDAC6 shRNA cells (BBP and DBP: 0/6 mice each). Thus, BBP and DBP induced metastatic tumor growth of estrogen-responsive breast epithelial stem cells. The results also indicate that the expression of HDAC6 plays an important role in phthalate-induced tumor growth.
DISCUSSION

In this study, we investigated the biological effects and gene expression induced by two phthalate compounds, BBP and DBP, in an estrogen-responsive breast stem cell line, R2d. Thus, similar to estrogen, BBP and DBP have the ability to promote tumor growth and metastasis. The results confirm that the tumorigenesis induced by phthalates is dependent on estrogen receptor with HDAC6 as a pivotal factor, leading to activation of upstream and downstream pathways. Our results affirm the effects of phthalate as an endocrine disruptor capable of activating the epigenetic factor, HDAC6, in breast stem cells. Moreover, phthalates may also induce EMT in breast stem cells.

In this study, it was found that phthalates, similar to estrogen, can induce EMT in breast stem cells and promote metastasis. This finding is in agreement with a previous report showing that BBP enhances cell migration in a different breast epithelial cell line, MCF-10F (Fernandez and Russo, 2010). We also found that ERs were responsible for phthalate-induced EMT in breast stem cells. Therefore, the estrogenic effects of phthalates contribute to activities that promote tumorigenicity. Figure 7 presents a simplified hypothetical mechanism of phthalate-induced HDAC6 expression mediated by the ER/EGFR/PKA/AP-2a pathway and leading to vimentin expression that involves Akt, GSK3β, and β-catenin subsequent to HDAC6 activation. Our study focused on the expression of HDAC6, finding that it is essential for phthalate-induced EMT. This finding is supported by another study showing that HDAC6 is required for TGF-α-induced transition of the epithelial-like phenotype into a mesenchymal phenotype via the SMAD3 signaling pathway (Shan et al., 2008) in various lung cancer cell lines. These findings are in agreement with an earlier study showing that ERs are interaction binding receptors with phthalates (Moore, 2000). ERs were reported to regulate cell motility through mediating HDAC6 signaling (Saji et al., 2005). Furthermore, our earlier study states that the major effect of HER2/neu expression is to upregulate HDAC6. Enhanced tumor growth may be mediated through the action of EGFR/HER2 heterodimerization (Hirsch et al., 2009). Although HDAC6 can regulate different signaling pathways in different cell types, all these reports indicate that HDAC6 plays a very important role in tumor growth, invasion, migration, and EMT induced by hormones such as TGF-α, estrogen, and phthalates.

We next attempted to elucidate the mechanisms of phthalate action. First, we tested the involvement of AP-2a and EGFR. Knockdown of AP-2a with siRNA induces a more differentiated phenotype and reduces tumor cell proliferation (Schulte et al., 2008). Interestingly, AP-2a has been reported to bind to the TGF-α promoter (Berkowitz et al., 1997), and TGF-α regulates EMT (Brandl et al., 2010) and cell proliferation (Casey et al., 2007). These observations suggest that AP-2a may be an important transcription factor for EMT regulation. We also investigated the possible involvement of the Akt pathway and β-catenin in vimentin expression. Akt phosphorylation may lead to phosphorylation and inactivation of GSK3β, which results in the localization of β-catenin to the nucleus and activation of TCF-4/LEF1. Subsequently, the binding of TCF-4/LEF1 to the vimentin promoter induces vimentin expression, which plays a role in EMT (Lee and Han, 2009; Li and Zhou, 2011). Our current results showed that phthalate treatment increased the level of the HDAC6/PP1 complex and decreased the level of the Akt/PP1 complex, leading to phosphorylation of Akt. This result is consistent with an earlier study showing that an HDAC inhibitor disrupts the PP1/HDAC complex, resulting in enhanced association of Akt with PP1 and thereby causing Akt dephosphorylation (Canettieri et al., 2003). Therefore, HDAC6 may be involved in a signaling through the EGFR/Akt pathway because the EGFR activation can also enhance Akt phosphorylation.

Sequential actions of Akt activation (phosphorylation), GSK3β inactivation, and accumulation of β-catenin in the nucleus (Monick et al., 2001) have been demonstrated to activate...
other genes such as c-myc that are involved in carcinogenesis (Pennis, 1998). Whether phthalates can induce the expression of these genes via a similar mechanism will be an interesting subject for future exploration. Taken together, our data suggest that some phthalates, such as BBP and DBP, regulate EMT to promote the proliferation and metastasis of breast cancer cells and subsequent tumor development by upregulating HDAC6. The mechanisms of action of these chemicals are likely to involve the HDAC6/PP1/Akt/β-catenin pathway. These results also suggest that HDAC6 is a promising chemopreventive and therapeutic target for breast cancer.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


