Phthalates induce proliferation and invasiveness of estrogen receptor-negative breast cancer through the AhR/HDAC6/c-Myc signaling pathway

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ABSTRACT The environmentally present group of chemical phthalates, or phthalate esters, has been recognized as a rising threat to public health, including cancer. While most studies have addressed the estrogenic effects of phthalates in malignancies of the breast and the prostate, little is known about their role in the etiology of hormone-independent cancer. Here we show that treatments with the phthalates n-butyl benzyl phthalate (BBP) and dibutyl phthalate (DBP) at 1 μM induced proliferation (BBP, 3.2-fold; DBP, 3.2-fold), migration (BBP, 2.6-fold; DBP, 2.6-fold), invasion (BBP, 2.7-fold; DBP, 3.1-fold), and tumor formation (EC50: BBP, 0.12 μM; DBP, 0.22 μM) in estrogen receptor (ER)-negative breast cancer cells (MDA-MB-231). We further demonstrate that phthalates stimulated the cell surface aryl hydrocarbon receptor (AhR) and triggered the downstream cyclic AMP (cAMP)-PKA-CREB1 signaling cascade. The pathway led to increased expression of HDAC6, which facilitated nuclear assembly of the β-catenin-LEF1/TCF4 transcriptional complex and transactivation of the c-Myc oncogene. This nongenomic pathway emanated from the phthalate-induced AhR promoted tumorigenesis of ER-negative breast cancer. Collectively, our findings revealed a novel oncogenic mechanism of phthalates in breast cancer independent from their estrogenic activities.—Hsieh, T.-H., Tsai, C.-F., Hsu, C.-Y., Kuo, P.-L., Lee, J.-N., Chai, C.-Y., Wang, S.-C., Tsai, E.-M. Phthalates induce proliferation and invasiveness of estrogen receptor-negative breast cancer through the AhR/HDAC6/c-Myc signaling pathway. FASEB J. 26, 778–787 (2012). www.fasebj.org

Key Words: environmental hormone · aryl hydrocarbon receptor · histone deacetylase 6 · ER-negative · nongenomic

Phthalate esters, such as n-butyl benzyl phthalate (BBP) and dibutyl phthalate (DBP), are widely used plasticizers. They are ubiquitously present in the environment, in products including cosmetics, hair sprays, and nail polishes. Moreover, phthalates have been found in medical products, toys, and food wrappers and therefore can be absorbed through the skin or ingested (1–2). Several studies have demonstrated that phthalate exposure is associated with an increased risk of breast cancer and resistance to endocrine therapy (3–5). Most studies about the mechanisms of phthalate-induced breast cancer focus on its estrogenic activity, which causes aberrant endocrine functions of steroid receptors. Intriguingly, numerous in vivo and in vitro studies have shown that phthalates such as BBP exhibit only a weak estrogenic activity in breast cancer cells, suggesting that phthalates may contribute to cancer development also through estrogen receptor (ER)-independent mechanisms (6–10). However, how phthalate exposure affects ER-negative breast cancer is poorly understood.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix family (11). In resting cells, AhR is sequestered by chaperone proteins in the cytoplasm. On binding with its stimulating ligands, such as phthalates and dioxins, AhR undergoes conformational change and is released from its cytoplasmic chaperones. The activated AhR, in turn, regulates gene expression through both nongenomic and genomic mechanisms (11–12). The former mechanism involves the nuclear translocation of AhR, which forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator and binds to AhR response elements on the promoters of target genes (13). In the nongenomic mechanism, AhR is translocated to the cytoplasmic membrane, where it
triggers a rapid signaling response through induction of intracellular calcium and cyclic AMP (cAMP; ref. 14), which ultimately leads to transcriptional activation in the nucleus (15). In either mechanism, the environmental ligands can stimulate AhR activation, which consequently regulates gene expression in the nucleus, and thus confers a significant effect on the pathophysiology of human cancer (16–17).

In the present study, we examine the molecular mechanism of phthalates on AhR activation in ER-negative breast cancer cells and unveil a novel mechanism by which phthalates activate the nongenomic function of AhR. The pathway leads to up-regulation of the HDAC6 and c-Myc oncogenes and promotes tumor progression of ER-negative breast cancer.

MATERIALS AND METHODS

Cell lines and chemicals

Human breast cancer cell lines MCF-7, T-47D, ZR-75-1, MDA-MB-231, MDA-MB-435, and SK-BR-3 were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM/F12 (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated in a humidified atmosphere with 5% CO2 at 37°C. Butyl benzyl phthalate (BBP; used at 1 mM) was purchased from Chemicon, Temecula, CA, USA. Cells were seeded onto a 96-well dish and incubated overnight, followed by incubation in serum-free medium for 24 h. Breast cancer cells were later exposed to phthalates for 24 h. The confluent monolayers were removed by swabbing and the invaded cells on the other side of the membrane were stained by crystal violet. For quantification, images were captured under a light microscope, and the cells were counted in 3 independent fields.

Flow cytometry staining

Cells were washed twice in ice-cold PBS and harvested with trypsin-EDTA (Invitrogen). After centrifugation, cell pellets were eluted by PBS, and ice-cold 75% ethanol was added, followed by incubation for 24 h at –20°C to fix the cells. The following antibodies were used for intracellular staining: anti-HDAC6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-acetylated α-tubulin (Sigma), and anti-c-Myc (Epitomics, Burlingame, CA, USA), according to the manufacturer’s recommendations. Cells were analyzed with an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA).

Reverse transcription-PCR and qPCR assay

Total RNA was isolated from breast cancer cell lines trizol (Invitrogen). RNA (2 μg) was used to synthesize cDNA with a Reverse Transcription System (Promega, Madison, WI, USA), and cDNAs were PCR-amplified with the primers of AhR (forward, 5'-TACTCTCGGCCGCACTTGG-3'; reverse, 5'-GCTGTGACGCTGATTCCG-3'), HDAC6 (forward, 5'-CCACACACGAGCAGCAGAGG-3'; reverse, 5'-ATCCCATTCCTGCCGTCCTGGG-3'), c-Myc (forward, 5'-ACACAAGAGCAGAGGAGG-3'; reverse, 5'-CTGTCGGCGTGGAGAGG-3'), β-actin (forward, 5'-AGATATGCGGCCTCTGGCTGC-3'; reverse, 5'-GCCTCGGCGTGGTGAGA-3'). The PCR products were separated in 2% agarose gels and stained with ethidium bromide. Quantitative PCR analysis was conducted using the ABI7500 system (Applied Biosystems, Foster City, CA, USA).

Immunoblotting and immunoprecipitation analyses

Treated cells were washed twice in ice-cold PBS and lysed using M-PER mammalian protein extraction reagent (Thermo Scientific, Franklin, MA, USA) and blotted onto PVDF membranes (Millipore, Bedford, MA, USA). The following antibodies were used for immunoblotting were purchased: anti-HDAC6, anti-AhR, anti-EGFR, antivimentin, antihistone 3 (Santa Cruz), anti-CREB1, anti-CREB1 (Ser133; Epitomics), and anti-β-actin and antiactietylated α-tubulin (Sigma). For immunoprecipitation studies, the lysate was precleared by incubating with protein-G beads (Roche, Indianapolis, IN) for 2 h at 4°C with rocking. The cleared lysate was then incubated with anti-IgG (Santa Cruz), anti-β-catenin (Epitomics), or anti-LEF1/TCF4 (Epitomics) in the presence of protein-G beads. The immunoprecipitated complexes were resolved in SDS gels and analyzed by immunoblotting.
Total internal reflection fluorescence (TIRF) microscopy

TIRF microscopes imaged and measured real-time cell membrane signaling of ~100 nm depth of live cells (20). To perform this analysis, breast cancer cells were transfected with pGFP-C1-AhR by LTI transfection reagent (Mirus, Madison, WI, USA). The pGFP-C1-AhR vector was obtained from Dr. Hsin-yu Lee (Life Science, National Taiwan University, Taipei, Taiwan; ref. 21). At 24 h after transfection, cells were cultured on coverslips and incubated overnight, followed by incubation in serum-free medium for 24 h. The cells were then stimulated with phthalates and subjected to continuous green fluorescent protein (GFP) analysis by TIRF microscopy. GFP epifluorescence intensity per cell with or without phthalate treatment was measured. All images of each experimental group were quantified by AxioVision 4.8 software analysis (Carl Zeiss, Oberkochen, Germany).

Immunofluorescence analyses

Cells were fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 for 20 min, and then blocked in PBS supplemented with 3% BSA. The following antibodies were used for immunofluorescence following the manufacturer’s recommendations: anti-HDAC6 (Santa Cruz Biotechnology), anti-acetylated α-tubulin (Sigma), and anti-β-catenin (Epitomics). Microscopic images were obtained using a laser scanning microscope.

Small interfering RNA (siRNA) transfection

The following siRNAs were used: control siRNA (sense: 5'-GCAUCAUGGUGCAUCAGA-3'; antisense: 5'-UCGAUGACCGUUACGAUG-3'), HDAC6 siRNA-1 (GenBank NM_000644; sense: 5'-GCAUAAUCUAUAUCGAAG-3'; antisense: 5'-UCUGAUAGAAGGAUAGC-3'), HDAC6 siRNA-2 (GenBank SASI_00048812; sense: 5'-CACUGAUGAGGCGCAUAAU-3'; antisense: 5'-AAUAGGCGCUACUGAG-3'), AhR (GenBank SASI_Hs02_003218); sense: 5'-GUCAGAGCUUGUUGGCU-3'; antisense: 5'-AGAAGCGAAAGGACCUAG-3'), CREB1 (GenBank NM_004379; sense: 5'-UCGAAGACAUCCAGAAG-3'; antisense: 5'-AUAUGGUAGCGUGUUG-3'). For transfection, cells were grown to ~80% confluence and then transfected with the siRNA (5 nM) using the LTI transfection reagent (Mirus) by following the manufacturer’s instructions. Cells were incubated with the siRNA-LTI complex for 24 h. The medium was then replaced with fresh serum-free medium for 24 h prior to phthalate treatments.

Cell fractionation

The nuclear, cytoplasmic, and membrane fractions were isolated using the CNMCS compartmental protein extraction kit (Biochain, Hayward, CA, USA) according to the manufacturer’s protocol.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) following the manufacturer’s protocol. RNAs in the chromatin immunocomplex were then extracted, and PCR was conducted with primers complementary to the HDAC6 promoter (forward: 5'-GGGCTCGTGGTATGGA-3', reverse: 5'-AGGGGCTGGTATGGA-3') and the c-Myc promoter (forward: 5'-ACCTACAGCTGGTGAGG-3', reverse: 5'-GGAATGATAGAGGCATAAGGAGTATC-3'). Amplified products were analyzed by electrophoresis in 1% agarose gels.

The amount of cAMP was measured using a cAMP-EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Cells were plated in 24-well plates. Following the treatment, cells were lysed with 100 mM HCl. For each sample well, data were recorded as picomoles cAMP per milligram and normalized to the appropriate control to calculate the percentage of stimulation or inhibition.

Animal studies and immunohistochemistry

All animal work was conducted according to the protocol approved by Kaohsiung Medical University Hospital Institutional Animal Care and Use Committee. Female nude mice (BALB/cAnN.Cg-Foxn1 /Crlnarl, 4–6 wk old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). MDA-MB-231 cells were infected with lentivirus derived from the pLKO.1-GFP vector and selected by puromycin to generate a stable line expressing GFP (MDA-MB-231-GFP). MDA-MB-231-GFP cells (2×10⁵) were subcutaneously injected into the flanks of these nude mice, and when the tumor size reached 30 mm², the mice were randomly divided into 3 treatment groups (normal saline, BBP, and DBP). DDP and DBP were intraperitoneally injected into animals at a dose of 800 mg/kg/d (22). Starting from 6 wk after implantation, tumor growth was monitored by the Ultra Sensitive Molecular Imaging System (Berthold Technologies, Bad Wildbad, Germany). In addition, tumor volumes were calculated by the following formula: t²/2, where t is the length and w is the width of the tumor. For immunohistochemical analysis of the xenograft tumors, 4-μm sections were deparaffinized with xylene and rehydrated with ethanol. Immunohistochemistry was conducted using the Dako LSAB kit (Dako, Hamburg, Germany) according to the manufacturer’s protocol. The stained slides were counterstained with Mayer’s hematoxylin, then mounted.

RESULTS

Phthalates promote in vitro tumor growth and invasion of cancer cells

To determine how MDA-MB-231 cells respond to phthalates, we examined cell growth in the presence of BBP, DBP, or the control vehicle. Treatment with the phthalates resulted in increased cell growth, as evidenced by measuring viable cells at 48 h (BBP, 1.2-fold; DBP, 1.1-fold) and 72 h (BBP, 1.3-fold; DBP, 1.4-fold) after the treatment (Fig. 1A). This growth-promoting effect of phthalates is dose dependent (data not shown). BrdU incorporation was also enhanced by the treatments (3.2-fold with BBP or DBP treatment; Fig. 1B), indicating that phthalates increase the proliferation of MDA-MB-231 cells. Consistently, treatment with BBP (Fig. 1C) and DBP (Fig. 1D) enhanced colony formation in a dose-dependent manner (EC₅₀: BBP, 0.12 μM; DBP, 0.22 μM). In addition to increased growth, cells treated with BBP and DBP had enhanced migratory activity, as measured by the numbers of cells migrating across a scratched area in a confluent cell monolayer. This wound-healing assay demonstrated...
that BBP- and DBP-treated cells had gained mobility (0.12 μM of BBP or 0.22 μM of DBP induced a 1.9-fold increase; 1 μM of BBP or DBP induced a 2.6-fold increase; Fig. 1E). Furthermore, treatment with the phthalates enhanced the ability of the cells invading through a matrix of extracellular matrix using the Boyd chamber invasion assay (0.12 μM of BBP induced 2.5-fold and 0.22 μM of DBP stimulated 2.4-fold; 1 μM of BBP or DBP stimulated a 2.7- or a 3.1-fold increase, respectively; Fig. 1E).

Phthalates mediate HDAC6 gene expression through the nongenomic function of AhR

Expression of AhR in a panel of ER-positive and ER-negative human breast cancer cell lines, as evaluated by quantitative PCR, showed that higher expression of AhR in ER-negative breast cancer cells than those in ER-positive breast cancer cell lines. We chose MDA-MB-231 for further mechanistic experiments because it expresses a high level of AhR (Fig. 2A). To further assess the molecular mechanism of the proliferative and invasive activities induced by phthalate treatments, we tested the response of AhR to phthalate treatments. We first used TIRF microscopy to analyze the real-time relocalization of AhR to the cell surface (see Materials and Methods for details). To visualize AhR in live cells, cells were transfected with GFP-AhR, followed by treatment with BBP or the vehicle control. Imaging analysis with TIRF microscopy showed that treatment with phthalates, but not the control vehicle, induced a pronounced membrane localization of AhR, as demonstrated by numerous dense spots in the membrane region on BBP treatment (Fig. 2B, top panel). A time-course analysis of the spot densities showed that the membrane localization of GFP-AhR peaked by 1 min after BBP induction, and the fluorescence intensity gradually vanished with time (Fig. 2B, bottom panel). To confirm the altered localization of endogenous AhR in response to phthalate treatment, cell lysates of the nuclear, cytoplasmic, and membrane fractions were examined for the distribution of AhR. In agreement with the TIRF results, phthalate treatment resulted in a significant increase of AhR protein in the membrane fraction (Fig. 2C). We next tested whether the cell surface relocation of AhR is associated with its activa-
tion by measuring the intracellular level of cAMP, which is known to be stimulated by AhR (15). As shown in Fig. 2D, BBP and DBP treatments indeed resulted in an acute accumulation of cAMP. The phthalate-induced increase of cAMP depended on the function of AhR, as inhibiting AhR by its antagonist (3′,4′-DMF) or by a specific siRNA (AhR siRNA) blocked cAMP accumulation (Fig. 2D). Activation of the cAMP pathway primarily leads to PKA activation, which then transactivates the transcription factor CREB1 by phosphorylation.

Figure 2. Phthalates mediate HDAC6 gene expression through the nongenomic function of AhR. A) Expression of AhR mRNA was determined by quantitative PCR in multiple breast cancer cell lines. B) GFP-AhR plasmid was transfected into MDA-MB-231 cells for 72 h. On exposure to BBP (1 μM), cells were monitored by continuous GFP analysis using real-time cell imaging microscopy. Recorded fluorescence intensity was calculated by the AxioVision 4.8 software; bottom panel shows plot. C) Localization of AhR in response to BBP or DBP treatment was assessed by cell fractionation and Western blot analysis. EGFR, vimentin, and histone 3 are markers of the membrane fraction, cytoplasmic fraction, and nuclear fraction, respectively. D) cAMP accumulation was induced by phthalate treatment, and the accumulation was abolished by AhR inhibitors. MDA-MB-231 cells were transfected with AhR siRNA (5 ng/μl) or pretreated with 3′,4′-DMF (AhR inhibitor, 1 μM) for 1 h, and then treated with 1 μM of phthalates for an additional 2 h. Accumulation of cAMP was measured. Data are means ± s.e. of 3 experiments. *P < 0.05 vs. untreated control; 1-tailed Student’s t test. E) Ser133 phosphorylation of CREB1 was induced by phthalate treatment. Cells were pretreated with 3′,4′-DMF (1 μM) or H89 (PKA inhibitor, 1 μM) for 1 h, followed by stimulation with either BBP or DBP (1 μM) for 2 h. Levels of total and Ser133-phosphorylated CREB1 (CREB1-P) were evaluated by Western blot analysis. F) Schematic diagram of the CREB1-binding element identified on HDAC6 promoter. G) CREB1 binds to the HDAC6 promoter on stimulation by phthalates. MDA-MB-231 cells were treated with or without 1 μM of phthalates for 2 h. Cells were then fixed, and the genomic DNA was sheared by sonication. CREB1-DNA complex was immunoprecipitated by an anti-CREB1 antibody. Isolated DNA was then amplified by primers flanking the tentative CREB1-binding sequence of the HDAC6 promoter. IgG was used as the control antibody. Input: internal control for the amount of genomic DNA used in the immunoprecipitation. H) RNA expression of the HDAC6 gene was enhanced by phthalates, which depended on the functions of AhR, PKA, and CREB1. Cells were transfected with the siRNA of AhR or CREB1 (5 ng/μl), or pretreated with 1 μM of the indicated inhibitors for 1 h. Cells were then treated with 1 μM of phthalates for 24 h, and expression of the HDAC6 gene was assessed by RT-PCR. Expression of β-actin was used as an internal control. I) Different cancer cell lines were pretreated with 3′,4′-DMF (1 μM) for 1 h, followed by stimulation with 1 μM phthalates for 24 h. RNA was isolated, and the amount of HDAC6 mRNA was determined by quantitative PCR. *P < 0.05.
ing it at serine 133 (23). In coherence with increased levels of cAMP, treatment with BBP or DBP enhanced phosphorylation of CREB1 at serine 133, which was abolished by 3',4'-DMF and the PKA inhibitor H89 (Fig. 2E). These results together establish that phthalates trigger the AhR-cAMP-PKA-CREB1 cascade and suggest a mechanism by which phthalates regulate gene expression in the nucleus. In searching for the target genes induced by this cascade, we identified the HDAC6 gene as a promising candidate because it is induced by CREB1, and the induction depends on the stimulation with the phthalates (see below). HDAC6 is a microtubule-associated deacetylase protein (24) that enhances cell motility (25) and tumor development (26). Our previous study also showed that the HDAC6 has an importation role in mediating tumorigenesis of tumor stem cells (27). Sequence analysis of the HDAC6 promoter revealed a consensus CREB1 response element (5'-GCAGT-3') in the upstream region of the HDAC6 promoter (Fig. 2F). Exposure to BBP or DBP induced dramatic recruitment of CREB1 onto the CREB1-binding site, while no CREB1 recruitment was detected on the HDAC6 promoter in untreated cells (Fig. 2G). We further show that the BBP- and DBP-induced CREB1 occupancy requires AhR, as depleting AhR using a specific siRNA blocked CREB1 recruitment. BBP or DBP treatment induced RNA expression of HDAC6 (Fig. 2H). The induction was blocked by the treatment with AhR inhibitors (3',4'-DMF and AhR siRNA), PKA inhibitor (H89), and siRNA of CREB1 (Fig. 2H), indicating a pathway in which phthalates induce gene expression of HDAC6 through the AhR-PKA-CREB1 cascade. Induction of HDAC6 expression by phthalates was not unique to MDA-MB-231 cells, as quantitative PCR showed that phthalate treatment also enhanced HDAC6 expression in SK-BR-3, another ER-negative breast cancer cell line (Fig. 2I).

**HDAC6 is required for phthalate-mediated cell growth and motility**

Corroborating these findings, expression of HDAC6 protein was induced on phthalate stimulation (Fig. 3A). The induced HDAC6 protein was functionally competent because it was able to catalyze deacetylation of α-tubulin, a known substrate of HDAC6, as shown by flow cytometry (Fig. 3B) and immunofluorescence (Fig. 3C). HDAC6 as a deacetylase plays a significant role in enhancing cancer cell growth and invasion (26). To investigate whether HDAC6 is required for the growth-promoting effects by phthalates, endogenous HDAC6 was silenced by specific siRNAs (Fig. 3D). The HDAC6-depleted cells were then treated with BBP and DBP. Depletion of HDAC6 blocked the phthalate-induced cell growth (Fig. 3E), migration (Fig. 3F), and invasiveness (Fig. 3G), demonstrating the critical role of HDAC6 in cell transformation induced by phthalates.

**Phthalates mediate c-Myc gene expression through HDAC6**

HDAC6 facilitates nuclear translocation of the β-catenin/LEF1/TCF4 transactivation complex, which then promotes c-Myc expression (28–29). Consistently, cell fractionation experiments showed that on phthalate treatment, β-catenin was increased in the nucleus and decreased from the cytoplasm (Fig. 4A). Notably, nuclear increase of β-catenin was blocked when cellular HDAC6 was depleted. This finding is further supported by confocal immunofluorescence microscopy, which demonstrated that the nuclear translocation of β-catenin was induced by BBP or DBP, and that depleting HDAC6 blocked the translocation (Fig. 4B). Moreover, reciprocal immunoprecipitation experiments demonstrated that phthalate treatment increased complex formation between β-catenin and LEF1/TCF4 (Fig. 4C), which was accompanied by the enhanced occupancy of LEF1/TCF4 on the c-Myc promoter (Fig. 4D and ref. 29). Consistently, RNA expression of c-Myc was induced by phthalate treatment, and the induction was abolished by depleting HDAC6 (Fig. 4E, F).

**In vivo tumor growth is mediated by phthalates**

To test the importance of c-Myc in phthalate-induced tumor growth, MDA-MB-231 cells stably expressing GFP were subcutaneously implanted in nude mice. The animals were then treated with BBP, DBP, or the control vehicle via intraperitoneal injection, and the growth of the xenograft tumors was monitored by whole-body bioluminescence (Fig. 5A). Phthalate treatments significantly increased tumor growth (Fig. 5B). Body weight monitoring showed no sign of major toxicity associated with the treatment (Fig. 5C). Consistent with the finding that phthalates induced HDAC6 and c-Myc expression in vitro, elevated expression of these two genes was detected by immunohistochemistry in tumor tissues treated by BBP and DBP, in comparison with the vehicle-treated tumors (Fig. 5D). Taken together, these results recapitulate the finding that phthalates promote tumor development of breast cancer through a signaling cascade to enhance tumor expression of HDAC6 and c-Myc.

**DISCUSSION**

Although numerous studies have demonstrated the involvement of phthalates in breast cancer, the majority of these studies focused on the estrogenic potential of phthalates in ER-positive cancer cells (such as MCF-7), while the ER-independent functions of phthalates are poorly understood. AhR itself is a potential drug target of breast cancer (30–31). Activation of AhR by benzo-[a]-pyrene, an environmental carcinogen, increases the invasion of ER-negative breast cancer (32). As depicted in Fig. 6, in this study we showed that AhR in the ER-negative MDA-MB-231 cells responded to phthalate stimulation by enhanced membrane translocation. The activated AhR triggered a nongenomic cascade, which
led to cAMP/PKA activation, CREB1 activation, transactivation of the HDAC6 promoter, and enhanced expression of key regulators of cancer development. HDAC6 is a known transcriptional target of ER (25). Our study also demonstrated that HDAC6 expression is induced by estrogen in breast cancer stem cells (27) and that activation of HDAC6 plays an important role in tumorigenesis (26). In ER-positive breast cancer cells, HDAC6 expression promotes cell migration and the response to endocrine therapy (25, 33). Our current study identifies HDAC6 as a critical transcriptional activator downstream of AhR in ER-negative breast cancer cells. Our finding is supported by a genome-wide study identifying HDAC6 promoter as a transcriptional target of CREB1 (34). We have provided evidence that the enhanced HDAC6 expression is responsible for the increased nuclear function of β-catenin to induce c-Myc expression, resulting in the promotion of proliferation and motility in ER-negative breast cancer cells exposed to phthalates. Our results

Figure 3. Role of HDAC6 in cancer cells treated with phthalates. A) Detection of HDAC6 and acetylated α-tubulin (Ac-tubulin) by immunoblotting (top 3 blot panels) and detection of HDAC6 mRNA by RT-PCR (bottom 2 blot panels) in cells treated with or without 1 μM phthalates. β-Actin was used as a loading control. B, C) HDAC6 and acetylated α-tubulin were detected by flow cytometry (B) and immunofluorescence (C) in cells treated with or without 1 μM phthalates. D) HDAC6 protein was detected in cells transfected with HDAC6 siRNA-1 (5 ng/μl) and HDAC6 siRNA-2 (5 ng/μl) compared to control siRNA (5 ng/μl) cells. At 48 h post-transfection, total protein was extracted and subjected to Western blot analysis. E) Cells transfected with 2 different siRNAs of HDAC6, siRNA-1 and siRNA-2, or the control siRNA were treated with the indicated phthalates (1 μM), and cell viability was assessed by XTT assays. F, G) Cells treated as in panel E were assessed for the activity in mobility (F) and invasion (G). Data are means ± SE of 3 experiments. *P < 0.05 vs. untreated control; 1-tailed Student’s t test.
Figure 4. Phthalates mediate c-Myc gene expression through HDAC6. A) Subcellular distribution of β-catenin in MDA-MB-231 cells treated with and without phthalates (1 μM) and HDAC6 siRNA-1 (5 ng/μl) was assessed by Western blot analysis. B) Cells transfected with HDAC6 siRNA were stimulated with 1 μM of either BBP or DBP for 24 h. Localization of β-catenin was then analyzed with fluorescence microscopy. Intensity of β-catenin in the nucleus was calculated by ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) and plotted. C) Cells were treated with 1 μM phthalates for 24 h, and cell lysates were incubated with anti-β-catenin or anti-LEF1/TCF4 antibody in the presence of protein G-Sepharose beads. Coimmunoprecipitated (IP) β-catenin and LEF1/TCF4 were examined by immunoblotting (IB). D) Cells were treated with or without 1 μM phthalates for 24 h. ChIP was conducted with an anti-LEF1/TCF4 antibody or an unrelated control antibody (IgG). Precipitated DNA was then amplified with primers specific for c-Myc. E, F) c-Myc gene expression in cells treated with and without phthalates was evaluated by quantitative PCR (E) and flow cytometry using an anti-c-Myc antibody (F). For these experiments, cells were transfected with HDAC6 siRNA followed by stimulation of cells with either 1 μM BBP or DBP for 24 h. Black, control; green, BBP; blue, DBP; red, HDAC6 siRNA-1; yellow, HDAC6 siRNA-1+BBP; brown, HDAC6 siRNA-1+DBP.
highlight the pivotal role of HDAC6 in phthalate-induced tumor growth and invasiveness through an ER-independent mechanism.

In summary, we have shown a novel signaling pathway through which phthalates act to promote tumor growth of ER-negative breast cancer cells. This finding will advance our understanding in the molecular impact of the environmental phthalates on breast cancer and help develop approaches for prevention and treatment of ER-negative breast cancer.

The authors thank the Center for Resources, Research, and Development of Kaohsiung Medical University for support with instrumentation, the Laboratory Animal Center of Kaohsiung Medical University for assistance with animal experiments, and the National RNAi Core Facility for providing the shRNAs. The authors also thank Dr. Shau-Ku Huang and Kazunari K. Yokoyama for critical review and valuable suggestions. This work was supported in part by the National Science Council, Taiwan (grants MY3, 99-2628-B-037-009-MY3), the Ministry of Education, Taiwan (grant KMU-EM-99-3), and the Kaohsiung Medical University (Hospital) Research Fund (grants KMUH 97-7K08, KMUH 98-8R19, KMUH 99-9F04, KMUH 99-9R30, and KMUER-004; to E.M.T). This study was also supported by the Susan Komen Breast Cancer Research Award (KG080540) and the startup fund of the University of Cincinnati (to S.C.W).

REFERENCES

9. Monteiro, P., Gilot, D., Le Ferrec, E., Rauch, C., Lagadic-

Received for publication June 28, 2011. Accepted for publication October 20, 2011.