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Xenoestrogens down-regulate aryl-hydrocarbon receptor nuclear translocator 2 mRNA expression in human breast cancer cells via an estrogen receptor alpha-dependent mechanism

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ABSTRACT

Environmental chemicals with estrogenic activity, known as xenoestrogens, may cause impaired reproductive development and endocrine-related cancers in humans by disrupting endocrine functions. Aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2) is believed to play important roles in a variety of physiological processes, including estrogen signaling pathways, that may be involved in the pathogenesis and therapeutic responses of endocrine-related cancers. However, much of the underlying mechanism remains unknown. In this study, we investigated whether ARNT2 expression is regulated by a range of representative xenoestrogens in human cancer cell lines. Bisphenol A (BPA), benzyl butyl phthalate (BBP), and 1,1,1-trichloro-2,2-bis(2-chlorophenyl-4-chlorophenyl)ethane (*o,p′*-DDT) were found to be estrogenic toward BG1Luc4E2 cells by an E-CALUX bioassay. ARNT2 expression was downregulated by BPA, BBP, and *o,p′*-DDT in a dose-dependent manner in estrogen receptor 1 (ESR1)-positive MCF-7 and BG1Luc4E2 cells, but not in estrogen receptor-negative LNCaP cells. The reduction in ARNT2 expression in cells treated with the xenoestrogens was fully recovered by the addition of a specific ESR1 antagonist, MPP. In conclusion, we have shown for the first time that ARNT2 expression is modulated by xenoestrogens by an ESR1-dependent mechanism in MCF-7 breast cancer cells.

1. Introduction

Environmental chemicals with estrogenic activity, known as xenoestrogens, are currently the largest group of known endocrine disruptors (EDs) (Welshons et al., 2003). Over the past few decades, a considerable number of publications have indicated that maternal exposure to EDs may cause impaired reproductive development and endocrine-related cancers in humans by disrupting endocrine functions. Concerns have been focused on a variety of environmental chemicals, including bisphenol A (BPA), phthalates, organochlorine pesticides, dioxin and polychlorinated biphenyls and their hydroxylated metabolites (OH-PCBs). However, the findings remain controversial (Safe, 2004; Sharpe and Irvine, 2004; Sikka and Wang, 2008). An excellent example of an ED is diethylstilbestrol (DES), a synthetic estrogen that was administered to pregnant women to prevent miscarriage during the 1940s and 1970s. It is currently well understood that prenatal exposure to DES may be associated with adverse pregnancy outcomes, genital tract abnormalities, infertility, and vaginal and cervical cancers (Hatch et al., 2010; Ma, 2009).

Aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2) is a member of the basic helix-loop-helix Per-ARNT-SIM (bHLH-PAS) family of transcription factors (Hirose et al., 1996) and acts as a common obligate partner for several other members of the family, including aryl hydrocarbon receptor (AHR) and hypoxia-inducible factor (HIF)-1α (Hankinson, 2008; Sekine et al., 2006). ARNT2 knockout mice suffer severe developmental defects and die shortly after birth (Hosoya et al., 2001). Similar findings were observed in the zebrafish (Hsu et al., 2001). Although many of the functions of ARNT2 remain unknown, it is believed that ARTN2 may play important roles in tumor angiogenesis (Maltepe et al., 2000) and many physiological pathways, including the responses to environmental contaminants, oxygen deprivation, biological rhythms, angiogenesis, and neuronal development (Hill et al., 2009). In addition, a recent epidemiological study found that ARNT2 expression was correlated with the prognosis of breast cancer patients by participating in the metabolism of certain environmental chemicals, indicating potential interactions between ARNT2 and estrogen receptor (ER) signaling pathways (Martinez et al., 2008).

In this study, we investigated the effects of xenoestrogens on ARNT2 expression in two estrogen-dependent cancer cell lines,

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MCF-7 human breast cancer cells and BG1Luc4E2 human ovarian cancer cells, and one estrogen-independent cancer cell line, LNCaP human prostate cancer cells. The estrogenic activities of a range of representative EDs were measured using an estrogenic chemically activated luciferase gene expression (E-CALUX) bioassay. We also examined the mechanism of ARNT2 regulation, in terms of the ER-dependent activation pathway.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO) and 17β-estradiol (E2) were obtained from Sigma Chemical Co. (St. Louis, MO). 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP) was obtained from Tocris (Ellisville, MO). DMSO was used as the primary solvent for all chemicals, and the DMSO solutions were further diluted in cell culture media for treatments. The final concentrations of DMSO in the media did not exceed 0.1% (vol/vol).

BPA, benzyl butyl phthalate (BBP), di-*n*-butyl phthalate (DBP), and di(2-ethylhexyl) phthalate (DEHP) were obtained from Wako Industries (Osaka, Japan). 2,3,3′,4′,5-Pentachloro-4-biphenylol (OH-PCB 107), 2,2′,3,4′,5,5′-hexachloro-4-biphenylol (OH-PCB 146), and 2,2′,3,4′,5,5′,6-heptachloro-4-biphenylol (OH-PCB 187) were obtained from Wellington Laboratories (Guelph, ON, Canada). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (Cambridge, MA). 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (*p*,*p*′-DDT) and 1,1,1-trichloro-2,2-bis(2-chlorophenyl-4-chlorophenyl)ethane (*o*,*p*′-DDT) were obtained from AccuStandard (New Haven, CT).

2.2. Cell culture

MCF-7 and LNCaP cells were obtained from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). The BG1Luc4E2 cells used in the E-CALUX bioassay were a gift from Dr. M. Denison (University of California, Davis, CA). The estrogen receptor 1 (ESR1)-positive BG1Luc4E2 cells are BG-1 ovarian cancer cells stably transfected with a luciferase reporter gene under the control of estrogen response element (ERE) that is responsive to the exposure to estrogen or estrogenic chemicals (Rogers and Denison, 2000). MCF-7, LNCaP, and BG1Luc4E2 cells were maintained in RPMI 1640 medium (Wako, Osaka, Japan) containing 10%, 10%, and 8% fetal bovine serum (FBS) (Mediatech, Herndon, VA), respectively. All of the cells were grown at 37 °C in a 5% CO_2 humidified incubator. For growth under steroid-free conditions, the cells were seeded in phenol red-free DMEM (MP Biomedicals, Solon, OH) containing 5% charcoal/dextran-treated FBS (Hyclone, Logan, UT). All the culture media contained 100 U/ml penicillin/streptomycin and 2 mmol/L L-glutamine (Mediatech, Herndon, VA).

2.3. Luciferase assay

BG1Luc4E2 cells were plated in 96-well plates (4×10^4 cells/well) and cultured under steroid-free conditions for 24 h, before exposure to EDs for 24 h. After removal of the medium, the plates were rinsed with phosphate-buffered saline (Gibco, Grand Island, NY), and the cells were lysed with 20 μl/well lysis buffer (Promega, Madison, WI). The luciferase activity was measured in an AB-2100 luminometer (Atto, Tokyo, Japan) after the addition of 100 μl/well luciferase assay reagent (Promega) and expressed as relative light units. Luciferase induction was calculated as a percentage of the vehicle control by setting the induction by DMSO at 100%. The half-maximal effective concentration, EC_{50}, was calculated for each test compound using a previously described method (Alexander et al., 1999).

2.4. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA for real-time RT-PCR was isolated from the three cell lines after treatment with the EDs for 24 h using an RNeasy Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Quantification and quality assessment of the isolated RNA samples were performed and verified using an Agilent Bioanalyzer 2100 and an RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA) in accordance with the manufacturer's instructions. RNA (9 μg) was transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The resulting cDNA (1 μl) was amplified in triplicate using TaqMan® Gene Expression Master Mix (Applied Biosystems) in accordance with the manufacturer's instructions. TaqMan® Gene Expression Assays (Applied Biosystems) used in this study were Hs00208298_m1 for *ARNT2*, Hs00420042_m1 for PDZ domain containing 1 (*PDZK1*), and Hs00266705_g1 for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The amplification reaction was performed in an ABI PRISM 7000 Sequence Detector (Applied Biosystems) under the following cycling conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The gene expression levels were calculated based on the threshold cycle using Sequence Detection System Software (Applied Biosystems). The gene expression was normalized by the GAPDH expression and set to 1 for the control DMSO-treated cells.

2.5. Western blot analysis

To evaluate the effects of EDs exposure on ARNT2 protein expression, Western blot was performed using the polyclonal anti-ARNT2 M-165 antibody from Santa Cruz Biotechnology (sc-5581, 1:500 dilution, Santa Cruz, CA). MCF-7 cells (2.5×10^6) treated with EDs were lysed using RIPA buffer solution (Santa Cruz Biotechnology). After boiling at 99 °C for 5 min, the protein samples were resolved by SDS polyacrylamide gel electrophoresis on a 10% gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After blotting in TBS with 5% non-fat dry milk–Tris buffered saline and 0.1% Tween, the membrane was probed with Actin H-196 (sc-7210, 1:500 dilution, Santa Cruz Biotechnology) or ARNT2 M-165 primary antibody. Blots were then incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (ECL plus Western blotting reagent pack, RPN2124, 1:10,000 dilution, GE Healthcare UK, Buckingham, England). The immune complex was detected with the Amersham ECL Plus™ Western Blotting Detection System (RPN2132, GE Healthcare UK). The blots were exposed to Hyperfilm (Amersham Pharmacia Biotech), and bands were quantified with ImageJ densitometry software (National Institutes of Health, Bethesda, MD).

2.6. Statistical analysis

All experiments in this study were performed in triplicates to test the reproducibility of the results. Quantitative data were expressed as the means ± SD. The statistical significance of differences between values was assessed using a two-tailed Student's *t*-test. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Estrogenic activities of EDs toward BG1Luc4E2 cells

The effects of E2 and several EDs on ESR1-mediated luciferase induction were measured by an E-CALUX bioassay in the human ovarian cancer cell line BG1Luc4E2 (Fig. 1 and Table 1). E2 significantly increased the ESR1-induced luciferase activity in a dose-dependent manner compared with the control DMSO-treated

Table 1
Estrogenic activity of EDs in the E-CALUX bioassay.

Chemicals	LOEC (M)	EC_{50} (M)	MOEC (M)	% of control (max)
E2	4.1×10^{-13}	4.8×10^{-13}	1×10^{-10}	263
BPA	1.23×10^{-7}	2×10^{-7}	3.33×10^{-6}	220
BBP	1.23×10^{-7}	2.45×10^{-7}	1×10^{-5}	295
DBP	–	–	–	–
DEHP	–	–	–	–
o,*p*′-DDT	3.13×10^{-7}	8.92×10^{-7}	5×10^{-6}	285
p,*p*′-DDT	3.13×10^{-7}	nd	5×10^{-6}	175
OH-PCB107	1.4×10^{-7}	nd	1.4×10^{-7}	117
OH-PCB146	2.3×10^{-10}	nd	1.3×10^{-7}	122
OH-PCB187	4.8×10^{-8}	nd	2.4×10^{-8}	125
TCDD	4.96×10^{-11}	6.0×10^{-10}	1.55×10^{-7}	43

LOEC, lowest observed effect concentration; EC_{50}, half of maximum effect concentration; MOEC, maximum observed effect concentration; –, no effect observed; nd, not determined; M, mol/L.