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Ligand structure-dependent activation of estrogen receptor α/Sp by estrogens and xenoestrogens

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ABSTRACT

This study investigated the effects of E2, diethylstilbestrol (DES), antiestrogens, the phytoestrogen resveratrol, and the xenoestrogens octylphenol (OP), nonylphenol (NP), endosulfan, kepone, 2,3,4,5-tetrachlorobiphenyl-4-ol (HO-PCB-Cl_4), bisphenol-A (BPA), and 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) on induction of luciferase activity in breast cancer cells transfected with a construct ($pSp1_3$) containing three tandem GC-rich Sp binding sites linked to luciferase and wild-type or variant ERα. The results showed that induction of luciferase activity was highly structure-dependent in both MCF-7 and MDA-MB-231 cells. Moreover, RNA interference assays using small inhibitory RNAs for Sp1, Sp3 and Sp4 also demonstrated structure-dependent differences in activation of ERα/Sp1, ERα/Sp3 and ERα/Sp4. These results demonstrate for the first time that various structural classes of ER ligands differentially activate wild-type and variant ERα/Sp-dependent transactivation, selectively use different Sp proteins, and exhibit selective ER modulator (SERM)-like activity.

1. Introduction

Estrogen receptor α (ERα) and ERβ are the two major ER subtypes, and the classical mechanism of estrogen action involves ligand-induced dimerization of ER which interacts with estrogen responsive elements (EREs) in target gene promoters and results in transcriptional activation [1,2]. This latter process is complex and involves interactions of the ligand-bound receptor with nuclear coactivators and other coregulatory proteins and components of the basal transcription machinery [3,4].

Ligand-dependent activation or inhibition of ER-dependent transactivation depends on several factors including ligand structure, cell/tissue-specific expression coactivators/coregulatory proteins, gene promoter and cell context [3]. The development of selective ER modulators (SERMs) such as tamoxifen and raloxifene for treatment of breast cancer and other hormone-related problems is due to this complex pharmacology in which individual SERMs exhibit tissue-specific ER agonist or antagonist activities [5–7]. Several *in vitro* assays for estrogenic activity using wild-type and variant forms of ERα and ERE-promoter-reporter constructs can distinguish between 17β-estradiol (E2) and different SERMs such as tamoxifen, raloxifene and "pure" antiestrogens such as ICI 164,384 and ICI 182,780 [8,9]. Moreover, studies in this laboratory have shown that structurally-diverse synthetic industrial estrogenic compounds (xenoestrogens) differentially activate ERE-promoters in cells transfected with wild-type and variant ERα expression plasmids suggesting that these compounds also exhibit SERM-like activity [10–13].

E2-dependent transactivation through nuclear pathways also involves non-classical mechanisms where the liganded ER interacts with other DNA-bound transcription factors including specificity proteins (Sp), activator protein-1 (AP-1), nuclear factor κB (NFκB), and globin transcription factor (GATA) [14–17]. ERα/Sp-dependent transactivation is responsible for activation of several genes in breast cancer cells responsible for cell proliferation, cell signaling, and nucleotide metabolism [17]. Ligand-dependent activation of ERα/Sp has been observed for both estrogens and antiestrogens such as 4'-hydroxytamoxifen (4-OHT) and ICI 182,780; however, in studies using a construct ($pSp1_3$) containing three GC-rich Sp protein binding sites, activation by estrogens and antiestrogens

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requires different domains of ERα [17–20]. For example, E2 activates $pSp1_3$ in cells transfected with wild-type ERα or DNA binding domain (DBD) mutants of ERα containing deletions of zinc finger 1 (ERαΔZF1) or zinc finger 2 (ERαΔZF2), whereas ICI 182,780 or 4-OHT activate ERα but not the DBD mutants [19,20]. $pSp1_3$ was not activated in cells transfected with ERβ [19].

In this study, we investigated the structure-dependent activation of ERα/Sp1 by a series of xenoestrogens including octylphenol (OP), nonylphenol (NP), endosulfan, kepone, 2,3,4,5-tetrachlorobiphenyl-4-ol (HO-PCB-Cl_4), bisphenol-A (BPA), and 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). E2, diethylstilbestrol (DES), 4-OHT, resveratrol (a phytoestrogen), and ICI 182,780 were also used as reference compounds for the study. With the exception of resveratrol, all compounds induced transactivation in breast cancer cells transfected with ERα and $pSp1_3$; however, activation of $pSp1_3$ in cells transfected with variant forms of ERα was structure-dependent. Moreover, using RNA interference that selectively decreases Sp1, Sp3 or Sp4 protein expression, we showed that xenoestrogens, E2 and antiestrogens selectively activate ERα/Sp1, ERα/Sp3 and ERα/Sp4.

2. Materials and methods

2.1. Chemicals, biochemicals and plasmids

Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Antibiotic antimycotic solution (AAS) (×100) was obtained from Sigma–Aldrich (St Louis, MO). The following test chemicals (and purities) were purchased from Sigma–Aldrich: E2 (≥98%), 4-OHT (≥98%), resveratrol (>99%), *p*-*t*-octylphenol (97%), *p*-nonylphenol (98%) and BPA (>99%). HO-PCB-Cl_4 was >98% pure as previously described [21]; HPTE (>98%) was synthesized as previously reported [22]. Kepone (98%) and endosulfan were purchased from Chem-Service (West Chester, PA). ICI 182,780 was provided by Dr. Alan Wakeling (Astra-Zeneca, Macclesfield, UK). Plasmid preparation kits were purchased from Sigma–Aldrich. All other chemicals were obtained from commercial sources at the highest quality available. Human ERα expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX); ERαΔZF1, ERαΔZF2, ERα(1–537), ERα(1–553), CFP-Sp1, YFP-ERα and CFP-YPF chimeras were made as previously reported [20,23].

2.2. Cells and transient transfection assays

MCF-7 and MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture Ham's F-12 (DME/F-12) (Sigma–Aldrich) supplemented with 2.2 g/l sodium bicarbonate, 5% FBS, and 5 ml/l AAS. Cells were cultured and grown in a 37 °C incubator with humidified 5% CO_2, 95% air. For transient transfection studies, MCF-7 or MDA-MB-231 cells were seeded in 12-well plates in DME/F-12 medium without phenol red supplemented with 2.2 g/l sodium bicarbonate, and 2.5% charcoal-stripped FBS. After 24 h, cells were transfected using the calcium phosphate transfection method with 350 ng of luciferase reporter construct ($pSp1_3$), 100 ng pcDNA3/His/lacZ (Invitrogen, Carlsbad, CA) as a standard reference for transfection efficiency, and 200 ng of the appropriate ER expression plasmid. The $pSp1_3$ construct and other plasmids containing E2-responsive GC-rich promoter inserts are not responsive to E2 even in MCF-7 cells and this is due to minimal TATA promoter and overexpression of the transfected construct which results in limiting levels of ERα [11,17–20]. E2- responsiveness requires cotransfection with ERα. Six hours after transfection, cells were shocked with 25% glycerol/PBS for 1 min, washed with PBS, and then treated with dimethylsulfoxide (DMSO, solvent) or different concentrations of estrogens, antiestrogens or xenoestrogens in DMSO for another 20–24 h. Cells were then washed twice in PBS and harvested with 100 μl of reporter lysis buffer (Promega Corp., Madison, MI). After two freeze–thaw cycles, cell lysates were centrifuged for 1 min at 16,000 × *g*, and the supernatant was used for determination of protein activity. Luciferase (Promega Corporation, Madison, MI) and β-galactosidase activity was determined using the Tropix Galacto-Light Plus assay system (Tropix, Bedford, MA). Light emission was detected on a lumicount micro-well plate reader (Packard Instruments, Meriden, CT), and luciferase activity was calculated by normalizing against β-galactosidase activity obtained from the same sample and compared with the DMSO control group (set at 100%) for each set of experiments.

2.3. RNA interference assay in cells transfected with iSp oligonucleotides

MCF-7 cells (5×10^4) were cultured in phenol red-free DME/F12 supplemented with 2.5% charcoal-stripped FBS without antibiotic in 12-well plates for overnight. siRNA (25 nM) was transfected by Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. After 6 h, the transfection medium was changed with fresh DME/F-12 and 2.5% serum without phenol red. The next day, following the manufacturer's instructions, Genejuice transfection reagent (EMD Biosciences Inc., San Diego, CA) was used to transfect cells with 200 ng of luciferase reporter construct ($pSp1_3$), 50 ng pcDNA3/His/lacZ (Invitrogen) as a standard reference for transfection efficiency, and 100 ng of the human ERα expression plasmid. Six hours later, cells were treated with DMSO or estrogens, antiestrogens, xenoestrogens in antibiotic-free, phenol red-free DMEM/F12 with 2.5% serum. Cells were harvested 48–54 h after siRNA transfection. Cell lysates were assayed for luciferase and β-galactosidase activity as described above.

The siRNA duplexes used in this study are indicated as follows. Silencer® Negative Control #1 siRNA purchased from Ambion (Austin, TX) was used as the non-specific control (iNS). The luciferase GL2 duplex (target sequence, 5′-CGT ACG CGG AAT ACT TCG A-3′) RNA from Dharmacon (Lafayette, CO) was used as the positive control in siRNA transfections. The siRNA oligonucleotides for Sp1, Sp3, and Sp4 were also obtained from Dharmacon as follows: Sp1, 5′-AUC ACU CCA UGG AUG AAA UGA dTdT-3′; Sp3, 5′-GCG GCA GGU GGA GCC UUC ACU dTdT-3′; and Sp4, 5′-GCA GUG ACA CAU UAG UGA GCdT dT-3′.

2.4. Western blot analysis

MCF-7 cells were seeded into six-well plates in DMEM/F12 supplemented with 2.5% charcoal-stripped FBS. The next day, cells were transfected with siRNA as described above. Fourty-eight hours after transfection, protein was extracted from the cells by harvesting in a high-salt lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, protease inhibitor cocktail (Sigma)] on ice for 45–60 min with frequent vortex and centrifugation at 20,000 × *g* for 10 min at 4 °C. Protein concentrations were determined using a Bio-Rad (Hercules, CA) protein assay reagent. Protein (60 μg) was diluted with Laemmli's loading buffer, boiled, and loaded onto 7.5% SDS-PAGE. Samples were resolved using electrophoresis at 150 V for 3–4 h and transferred (transfer buffer, 48 mM Tris–HCl, 29 mM glycine, and 0.025% sodium dodecyl sulfate) to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electrophoresis at 0.2 A for