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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-Cl₄), 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 (pSp1₃) containing three tandem GC-rich Sp binding sites linked to luciferase and wild-type or variant ER α . 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 ER α /Sp1, ER α /Sp3 and ER α /Sp4. These results demonstrate for the first time that various structural classes of ER ligands differentially activate wild-type and variant ER α /Sp-dependent transactivation, selectively use different Sp proteins, and exhibit selective ER modulator (SERM)-like activity.

Keywords

ER α /Sp; transactivation; xenoestrogens; 17 β -estradiol; antiestrogens

1. Introduction

Estrogen receptor α (ER α) and ER β are the two major ER sub-types, 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].

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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 ER α and ERE-promoter-reporter constructs can distinguish between 17 β -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 ER α 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 κ B (NF κ B), and globin transcription factor (GATA) [14–17]. ER α /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 ER α /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 (pSp1₃) containing three GC-rich Sp protein binding sites, activation by estrogens and antiestrogens requires different domains of ER α [17–20]. For example, E2 activates pSp1₃ in cells transfected with wild-type ER α or DNA binding domain (DBD) mutants of ER α containing deletions of zinc finger 1 (ER α Δ ZF1) or zinc finger 2 (ER α Δ ZF2), whereas ICI 182,780 or 4-OHT activate ER α but not the DBD mutants [19;20]. pSp1₃ was not activated in cells transfected with ER β [19].

In this study, we investigated the structure-dependent activation of ER α /Sp1 by a series of xenoestrogens including octylphenol (OP), nonylphenol (NP), endosulfan, kepone, 2,3,4,5-tetrachlorobiphenyl-4-ol (HO-PCB-Cl₄), 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 ER α and pSp1₃; however, activation of pSp1₃ in cells transfected with variant forms of ER α was structure-dependent. Moreover, using RNA interference that selectively decreases Sp1, Sp3 or Sp4 protein expression, we showed that xenoestrogens, E2 and antiestrogen selectively activate ER α /Sp1, ER α /Sp3 and ER α /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) (x100) was obtained from Sigma-Aldrich (St Louis, MO). The following test chemicals (and purities) were purchased from Sigma-Aldrich: E2 ($\geq$ 98%), 4-OHT ($\geq$ 98%), resveratrol ($>$ 99%), *p*-*t*-octylphenol (97%), *p*-nonylphenol (98%) and BPA ($>$ 99%). HO-PCB-Cl₄ 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 ER α expression plasmid was kindly provided by Dr. Ming-Jer Tsai (Baylor College

of Medicine, Houston, TX); ER α Δ ZF1, ER α Δ ZF2, ER α (1–537), ER α (1–553), CFP-Sp1, YFP-ER α 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/liter sodium bicarbonate, 5% FBS, and 5 ml/liter AAS. Cells were cultured and grown in a 37°C incubator with humidified 5% CO₂, 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/liter sodium bicarbonate, and 2.5% charcoal-stripped FBS. After 24 hr, cells were transfected using the calcium phosphate transfection method with 350 ng of luciferase reporter construct (pSp1₃), 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 pSp1₃ 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 ER α [11;17–20]. E2-responsiveness requires cotransfection with ER α . Six hr 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 hr. Cells were then washed twice in PBS and harvested with 100 μ L 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 hr, 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 (pSp1₃), 50 ng pcDNA3/His/lacZ (Invitrogen) as a standard reference for transfection efficiency, and 100 ng of the human ER α expression plasmid. Six hr 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 hr 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. Forty-eight hr 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% (vol/vol) 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 150V for 3 – 4 hr 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 approximately 12 – 16 hr. Membranes were blocked in 5% TBS-Tween 20-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, 5% nonfat dry milk] with gentle shaking for 30 min and incubated in fresh 5% TBS-Tween 20-Blotto with 1:1,000 (for Sp1 and Sp3), 1:500 (for Sp4), and 1:5,000 (for α-actin) primary antibody overnight with gentle shaking at 4°C. The primary antibodies for Sp1 (PEP2), Sp3 (D-20), and Sp4 (V-20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and antibody for α-actin was purchased from Sigma. Membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:5000) (Santa Cruz) for 3 – 6 hr at 4°C. Blots were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) and exposure on Image Tek-H x-ray film (American X-ray Supply). Band quantitation was performed by Image (NIH).

2.5 Fluorescence resonance energy transfer (FRET)

FRET was performed with the Stallion DDI workstation equipped with a xenon fluorescent light source (300 W) with rapid switching (<2 msec) between excitation wavelengths and two CoolSnap HQ cameras for simultaneous detection of two emission signals. Three types of images were collected for the FRET experiments. The first type was the CFP control (donor only) which was used to calculate the spectral bleed-through of CFP emission visible through the FRET filter set. This type of image was collected using the CFP filter set and the FRET filter set. The second type of image, the YFP control (acceptor only), was used to calculate the spectral bleed-through of normal YFP emission through the FRET filter set. This image was collected using the YFP filter set and the FRET filter set. The third type of image is the raw FRET image (FRETraw) representing the sample under analysis. This type of image was collected using the FRET, CFP and YFP filter sets. Corrected FRET (FRETc), was calculated with consideration of all three images [24] using the following equation:

$$\text{FRETc} = \text{FRETraw} - \text{Df/Dd[CFP]} - \text{Df/Da[YFP]}$$

where FRETraw, [CFP], and [YFP] are the signals visualized through the FRET, CFP, and YFP filter sets, respectively. The constants Df/Dd and Df/Da are the bleed-through constants describing donor emission visible in FRET channel and direct excitation of acceptor, respectively, calculated from the first and second type images. FRET efficiency was then calculated by comparing the constructs under investigation to the CFP-YFP chimera (positive control) [23].

2.6 Real-time PCR

MCF-7 cells (3×10^6) were seeded into six-well plates in DMEM/F-12 supplemented with 2.5% charcoal-stripped FBS. The next day, cells were transfected with non-specific siRNA or iSp1/iSp3/iSp4 as described above. Forty-eight hr after transfection, cells were treated with 10 nM E2, 20 µM HPTE, 5 µM kepone for 6 hr. Total RNA was isolated using the RNeasy Protection Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol and treated with RNase-free DNase set (QIAGEN). RNA was eluted with 50 µl RNase-free water. RNA was

reverse transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was carried out using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Each reaction contained 0.5 μ M primer and 2 μ l cDNA template and made up to 25 μ l. The relative quantitation of samples was carried out using comparative Ct method. TATA binding protein (TBP) was used for normalization. Cad primers were purchased from QIAGEN and TBP primers were synthesized by Integrated DNA Technologies (Coralville, IA). TBP (forward), 5'-TGC ACA GGA GCC AAG AGT GAA-3'; and TBP (reverse), 5'-CAC ATC ACA GCT CCC CAC CA-3'.

2.7 Statistical analysis

For transient transfection studies, results are expressed as means $\pm$ SD for at least three separate experiments for each treatment group. Statistical differences ($p < 0.05$) between control (DMSO) and treatment groups were determined by single factor ANOVA test. For FRET efficiency analysis, data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test at $p < 0.05$.

3. Results

3.1 Ligand structure-dependent activation of wild-type ER α /Sp

pSp1₃ is a plasmid containing three consensus GC-rich sites with a minimal TATA promoter linked to the luciferase gene. This construct is not inducible by E2 in ER-positive MCF-7 or ZR-75 cells and E2-responsiveness is only observed after cotransfection with ER α or variant forms of ER α [17–20;23;25;26]. This is due to overexpression of the construct where endogenous ER α in MCF-7 cells becomes limiting. The lack of responsiveness without cotransfection of ER α is also due to the minimal TATA promoter. This has proven to be an excellent model system for determining ligand structure-dependent requirements for different domains of ER α in transactivation studies in a breast cancer cell (ER-positive or ER-negative) context [10–12;17–20]. ERE-promoter-reporter constructs have been extensively used as models for understanding the classical ER α -mediated transactivation pathways, and pSp1₃ is a model for investigating mechanisms of ER α /Sp-mediated gene expression [17]. The effect of ligand structure on activation of ER α /Sp-dependent transactivation was investigated in MCF-7 cells transfected with pSp1₃ and treated with different concentrations of E2, DES, ICI 182,780 and 4-OHT (Figs. 1A and 1B). Both E2 and DES induced luciferase activity and similar results were observed for the antiestrogens 4-OHT and ICI 182,780. The induced transactivation for the antiestrogens was similar to previous studies using this same construct (pSp1₃) in both ER-positive and -negative cancer cell lines [20]. Several reports show that xenoestrogens and phytoestrogens activate gene expression in MCF-7 cells transfected with ERE constructs [10–13]; however, their activation of pSp1₃ has not previously been reported and is investigated in this study.

The results in Figure 1C summarize the concentration-dependent activation of pSp1₃ by two bisphenolic estrogenic compounds HPTE and BPA; both compounds induced luciferase activity, with HPTE being the more potent of the two bisphenols. Octyl- and nonylphenol (Fig. 1D) also induced transactivation in cells transfected with pSp1₃ and, among the bisphenolic and phenolics xenoestrogens, their induction response varied from 4- to 8-fold. A third phenolic compound HO-PCB-Cl₄ also induced approximately a 4-fold increase in luciferase activity, whereas resveratrol, a trihydroxystilbene analog was inactive in this assay (Fig. 1E). Both endosulfan and kepone are chlorinated caged xenoestrogenic compounds and the potency of endosulfan was similar to that observed for the phenolic/bisphenolic compounds (6.5-fold induction); however, a > 25 -fold increase in luciferase activity was observed in MCF-7 cells transfected with pSp1₃ and treated with kepone which induced the highest activity among all

the 12 compounds tested in this assay (Fig. 1F). All compounds induced a concentration-dependent induction of luciferase activity and, in subsequent studies, we used concentrations that induced maximal activity with no cytotoxicity.

3.2 Ligand structure-dependent activation of wild-type and mutant ER α /Sp

Previous studies showed that although E2 and antiestrogens activate wild-type ER α /Sp-dependent transactivation in MCF-7 cells transfected with pSp1₃; however, activation of deletion and point mutant forms of ER α (transfected) are ligand structure-dependent [19;20]. We therefore compared the effects of different structural classes of xenoestrogens and resveratrol on induction of luciferase activity in MCF-7 cells transfected with pSp1₃ and wild-type ER α (Fig. 2A), ER $\alpha\Delta$ ZF1 (Fig. 2B), ER $\alpha\Delta$ ZF2 (Fig. 2C), ER α (1–553) (Fig. 2D), and ER α (1–537) (Fig. 2E). These ER α mutants contained deletions of zinc finger 1 (amino acids 185–205), zinc finger 2 (amino acids 218–243), the F domain (amino acids 554–595), and the F domain plus amino acids in helix 12 of the E domain (amino acids 538–595), respectively. With the exception of resveratrol, all compounds induce transactivation in MCF-7 cells transfected with pSp1₃/ER α (Fig. 2A) as observed in Figure 1. The results in Figures 2B and 2C show that the antiestrogens ICI 182,780 and 4-OHT did not induce transactivation in MCF-7 cells transfected with pSp1₃ and ER $\alpha\Delta$ ZF1 or ER $\alpha\Delta$ ZF2 as previously reported [19;20]. In contrast, both E2 and DES significantly activated luciferase activity, and the xenoestrogens also induced transactivation and resembled E2. Resveratrol was also significantly active, and maximally induced responses were observed for HPTE, kepone and HO-PCB-Cl₄ in cells transfected with both zinc finger deletion mutants. The fold-induction responses were decreased in cells transfected with ER α (1–553) in which the F domain has been deleted; however, in contrast to a previous report [20], E2 and the remaining compounds all significantly induced transactivation (Fig. 2C), and HPTE, HO-PCB-Cl₄ and kepone were the most potent inducers. Major structure-dependent differences were observed in MCF-7 cells transfected with ER α (1–537); E2 and DES did not induce transactivation, whereas the antiestrogens, resveratrol and xenoestrogens were all inducers of luciferase activity with kepone, HO-PCB-Cl₄ and HPTE among the most active compounds. Since ER α (1–537) does not contain an intact helix-12 which is important for coactivator-ER α interactions, this suggests that other nuclear cofactors may be involved and this is currently being investigated.

We also investigated ligand structure-dependent activation of ER α /Sp-dependent transactivation in ER-negative MDA-MB-231 breast cancer cells transfected with pSp1₃, wild-type ER α or the same set of ER α mutants ER $\alpha\Delta$ ZF1, ER $\alpha\Delta$ ZF2, ER α (1–554) and ER α (1–537), respectively (Figs. 3A – 3E). The concentrations of xenoestrogens were initially optimized for this cell line, and the estrogens, antiestrogens and xenoestrogens all induced luciferase activity in cells cotransfected with pSp1₃ and wild-type ER α (Fig. 3A). The pattern of induction in MDA-MB-231 cells transfected with the zinc-finger mutants was similar to that observed in MCF-7 cells; E2 and DES but not the antiestrogens ICI 182,780 or 4-OHT induced activity, and with the exception of the alkylphenols, the xenoestrogens and resveratrol were all active with the latter compound exhibiting significantly higher activity in MDA-MB-231 than in MCF-7 breast cancer cells (Figs. 3B and 3C). In MDA-MB-231 cells transfected with the F domain deletion mutant [ER α (1–553)] (Fig. 3D), all compounds with the exception of the alkylphenols and endosulfan induced activity, and these results were similar to those obtained in MCF-7 cells (Fig. 2D) where only limited induction was observed for alkylphenols and endosulfan. Higher concentrations of these compounds may induce transactivation in MDA-MB-231 cells; however, this was not carried out due to cytotoxicity. The structure-dependent induction of luciferase activity in MDA-MB-231 cells transfected with ER α (1–537) (Fig. 3E) was also similar to that observed in MCF-7 cells (Fig. 2E) in which the xenoestrogens and resveratrol but not E2 or DES were active. These results demonstrate that xenoestrogens, resveratrol, antiestrogens and E2/DES differentially activate wild-type and variant ER α /Sp in

ER-negative MDA-MB-231 cells transfected with pSp1₃, and the pattern of activation of wild-type and variant ER α /Sp by xenoestrogens was similar in both MCF-7 and MDA-MB-231 cells.

3.3 Role of Sp1, Sp3 and Sp4 in activation of a GC-rich construct (pSp1₃)

Previous studies show that hormonal activation of other GC-rich promoters involves ER α /Sp1, ER α /Sp3 and ER α /Sp4 [25;26]. The role of individual Sp proteins on ligand structure-dependent activation of ER α /Sp in MCF-7 cells transfected with pSp1₃ was further investigated using RNA interference and small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4). Results illustrated in Figure 4A summarize Western immunoblot analysis of Sp protein levels in whole cell lysates from MCF-7 cells transfected with iSp1, iSp3, iSp4 and binary combination of these reagents. Since transfection efficiencies are in the range of 60–90%, the results demonstrate highly effective and specific Sp protein knockdown as previously reported using these same siRNAs [25–27]. The siRNAs were initially used to investigate the role of individual Sp proteins in mediating E2-, 4-OHT- and HPTE-mediated activation of pSp1₃ in MCF-7 cells and determine possible structure-dependent utilization of one or more Sp proteins. HPTE was selected as a prototypical xenoestrogen that induces transactivation in cells transfected with wild-type and variant ER α expression plasmids. In all experiments, iSp1, iSp3 and iSp4 decreased basal and ligand-induced activity and therefore the results are expressed as fold induction in order to directly compare the role of individual Sp proteins on ligand-dependent activation of ER α /Sp. Results in Figure 4B demonstrate that iSp1 alone or in combination with iSp3 or iSp4 completely decreased inducibility by E2. iSp4 alone or in combination with iSp1 or iSp3 decreased induction by E2, whereas iSp3 did not affect the induction response. These results suggest that ER α /Sp1 is the major complex activated by E2, and ER α /Sp4 also contributes to this response. In contrast, Sp3 did not significantly decrease E2 inducibility, suggesting that ER α /Sp3 does not play a role in hormonal activation of pSp1₃. These results were observed in several replicate experiments showing that E2-dependent activation of ER α /Sp followed the order ER α /Sp1 > ER α /Sp4 > ER α /Sp3. Using a similar approach, we also investigated HPTE- and 4-OHT-dependent activation of ER α /Sp (Figs. 4C and 4D, respectively) and the results suggest that ER α /Sp1 and ER α /Sp4 but not ER α /Sp3 play a role in 4-OHT/HPTE activation of pSp1₃. The major difference between E2 and 4-OHT/HPTE over several replicate experiments was the increased role of ER α /Sp4 for the latter two compounds.

The effects of several xenoestrogens on activation of ER α /Sp-dependent transactivation were also investigated in MCF-7 cells cotransfected with pSp1₃ and iSp1, iSp3 or iSp4 (Fig. 5 and Fig. 6). Figure 5 summarizes the effects of various iSps on the induction of luciferase activity in MCF-7 cells transfected with pSp1₃ and treated with DES, HO-PCB-Cl₄, BPA, NP and endosulfan (Figs. 5A – 5E, respectively). The results show that like E2, DES and HO-PCB-Cl₄ primarily activate ER α /Sp1 and also activate ER α /Sp4 (<ER α /Sp1) but not ER α /Sp3 (Figs. 5A and 5B). BPA, endosulfan and NP activate ER α /Sp1 and ER α /Sp4 but not ER α /Sp3 (Figs. 5C – 5E) and resemble 4-OHT in their differential activation of ER α /Sp. The effects of iSps on activation of pSp1₃ by ICI 182,780 (Figs. 6A and 6B) and kepone (Figs. 6C and 6D) were different from those observed for the other ER α agonists and antagonists. Induction of luciferase activity by ICI 182,780 was decreased 40 – 60% by individual and combined iSps (Fig. 6A); however, basal activity was decreased in the order iSp4 > iSp3 > iSp1, and the lowest basal activity was observed in cells transfected with iSp4 alone or in combination with other iSps. When the luciferase activity is plotted as fold induction, the results show that ICI 182,780 activates ER α /Sp1 and ER α /Sp3 since iSp3, iSp1 and iSp1 plus iSp3 significantly decreased transactivation (Fig. 6B). Similar results were observed for kepone (Figs. 6C and 6D), although neither iSp3 or iSp4 were as effective in decreasing fold inducibility by kepone compared to ICI 182,780. The results demonstrate that activation of ER α /Sp-dependent transactivation by

ICI 182,780 and kepone differs from the other estrogens, xenoestrogens and 4-OHT with respect to the role of Sp1, Sp3 and Sp4 in mediating this response.

3.4 Result of FRET studies

Previous studies showed that E2, 4-OHT and ICI 182,780 induced interactions of chimeric CFP-Sp1 and YFP-ER α in MCF-7 cells transfected with expression plasmids for these chimeras and analyzed by fluorescence resonance energy transfer (FRET) [23]. FRET efficiencies could be determined by comparing results to the YFP-CFP chimera which was used as a positive control [23]. Using this approach, we compared FRET efficiencies for E2 and selected xenoestrogens in MCF-7 cells transfected with CFP-Sp1 and YFP-ER α . Figure 7A illustrates the pseudocolor CFP and YFP fluorescence intensities in MCF-7 cells treated with DMSO or 10 μ M kepone. The FRET signal in the kepone-treated cells indicates an increase in FRET efficiency compared to DMSO and that kepone induces ER α -Sp1 interactions at a distance of < 10 Å and thereby allows energy transfer. The FRET efficiency values were determined 20 – 30 min after treatment with 10 nM E2, 25 μ M HO-PCB-Cl₄, 10 μ M kepone, and 75 μ M BPA, and FRET efficiencies were significantly increased compared to solvent (DMSO) control (Fig. 7B). These results demonstrate that xenoestrogens not only activate ER α /Sp-dependent transactivation but also induce ER α -Sp1 interactions in living breast cancer cells. Currently, we are investigating ligand structure-dependent differences in the rate of ER α interactions with Sp1, Sp3 and Sp4 using the FRET technique.

E2 induces carbamoylphosphate synthetase / aspartate transcarbamylase / dihydroorotase (CAD) gene expression in MCF-7 cells through interactions of ER α /Sp with proximal GC-rich motifs [28]. The role of Sp protein in mediating induction of CAD by three prototypical compounds, E2, HPTE and kepone, in MCF-7 cells is illustrated in Figure 7C. E2, HPTE, and kepone induced CAD gene expression, and induction was significantly inhibited in cells cotransfected with iSp1/iSp3/iSp4 (combined). This confirms that activation of CAD by E2 and xenoestrogens is also Sp-dependent, and current studies are investigating ligand structure-dependent activation and inhibition of other E2-response genes through the ER α /Sp pathway.

4. Discussion

E2 is the major endogenous hormone for both ER α and ER β , and there is increasing evidence that activation of both receptors is complex and dependent on multiple factors including relative expression of each specific ER-subtype and their corresponding coactivators and coregulatory proteins [1–4]. The complexity of estrogen signaling is also linked the multiplicity of signaling pathways which include both genomic and non-genomic mechanisms where the later pathway involves extranuclear interactions of ER with multiple cytosolic or membrane-associated proteins that cooperatively activate kinase signaling [29;30]. Genomic pathways of estrogen action include not only activation through direct binding of ER (dimeric) to EREs in target gene promoter but also activation of genes through interactions of ER with other DNA bound proteins including specificity proteins, AP1, GATA-1 and NF κ B [14–17]. There is also evidence showing that hormonal activation of genes through ERE promoter sequences is structure-dependent and the tissue specific ER agonist and ER antagonist activities of SERMs has led to their applications for treating breast cancer and other hormone-related diseases [5–7].

Several *in vitro* assays clearly distinguish between the activities of E2 and antiestrogens such as ICI 182,780 and tamoxifen to activate ERE-dependent genes/promoter constructs [8–13]. Structurally-diverse synthetic industrial estrogenic compounds (xenoestrogens) and phytoestrogens also differentially activate various ERE-dependent constructs and related genes [31–40], suggesting that these compounds also exhibit SERM-like activity. The genomic ER α /Sp1 pathway for activating E2-responsive genes containing GC-rich motifs is critical for

regulating growth-promoting genes and G₀/G₁ to S phase progression in MCF-7 cells [17], and this study was carried out to determine whether structurally-diverse xenoestrogens also differentially activate ER α /Sp. Previous studies showed that E2 induced transactivation in MCF-7, ZR-75 or MDA-MB-231 cells transfected with pSp1₃ and cotransfection with wild-type ER α or DBD deletion mutants [19;20], whereas ICI 182,780 or 4-OHT induced transactivation only in cells transfected with wild-type ER α . The pSp1₃ construct is a prototypical E2-responsive GC-rich promoter which has been used to investigate differences between E2 and the antiestrogens 4-OHT and ICI 182,780 to activate ER α /Sp in breast cancer cells. In this study, pSp1₃ was used to investigate the SERM-like activity of structurally-diverse xenoestrogens and resveratrol by determining the effects of cell context (MCF-7 vs. MDA-MB-231), wild-type and variant ER α and different Sp proteins (Sp1, Sp3 and Sp4) on their estrogenic activities. However, in all cases, the relative potencies of the xenoestrogens for activating ER α /Sp were greater than 1000-fold lower than E2. With the exception of resveratrol in MCF-7 cells, all compounds including the antiestrogens ICI 182,780 and 4-OHT, E2, DES and the xenoestrogens induced wild-type ER α /Sp-dependent transactivation in MCF-7 and MDA-MB-231 cells (Fig. 1 – Fig. 3). E2 and DES, but not ICI 182,780 or 4-OHT, induced transactivation in MCF-7 cells transfected with ER α Δ ZF1 or ER α Δ ZF2. The pattern of xenoestrogen activation of pSp1₃ in cells transfected with ER α , ER α Δ ZF1 and ER α Δ ZF2 (Fig. 2) was similar to that observed for E2 and DES, and the most active compounds were kepone, HPTE, HO-PCB-Cl₄, endosulfan and nonylphenol. Activation of ER α Δ ZF1/Sp or ER α Δ ZF2/Sp by xenoestrogens distinguished between these compounds and ICI 182,780 and 4-OHT which were inactive in cells transfected with the DBD mutants.

Previous studies [20] showed that E2 and the antiestrogens ICI 182,780 and 4-OHT differentially induced activity in MCF-7 cells transfected with pSp1₃ and the C-terminal deletion mutants ER α (1–553) and ER α (1–537). ER α (1–537) does not express the F domain and part of helix 12 which interacts with coactivators. Not surprisingly, E2 and DES did not induce activity in MCF-7 (Fig. 2E) or MDA-MB-231 (Fig. 3E) cells transfected with ER α (1–537) and pSp1₃; however, significant induction was observed for both 4-OHT and ICI 182–780. Moreover, in cells transfected with pSp1₃ and ER α (1–537), like the antiestrogens, the xenoestrogens and resveratrol also induced transactivation. Thus, deletion of a region of helix 12 in the C-terminal domain of ER α , which totally abrogates E2-responsiveness, does not affect xenoestrogen-induced transactivation in cells transfected with pSp1₃ demonstrating a fundamental mechanistic difference between activation of ER α /Sp by E2 and the xenoestrogens/resveratrol used in this study.

Thus, results of transfection studies using wild-type and variant ER α show that xenoestrogen activation of ER α /Sp required different domains of ER α than either E2/DES or ICI 182,780/4-OHT. Recent studies in ZR-75 cells showed that ER α /Sp-dependent activation of vascular endothelial growth factor receptor 2 (VEGFR2) by E2 was primarily dependent on ER α /Sp3 and ER α /Sp4 but not ER α /Sp1 [25]. Thus, activation of VEGFR2 by E2 required interactions of ER α with only two of the Sp proteins and, in this study, we investigated structure-dependent activation of ER α /Sp1, ER α /Sp3 and ER α /Sp4 by E2, antiestrogens and xenoestrogens. RNA interference was used to individually knockdown Sp1, Sp3 and Sp4 or their combinations (Fig. 4A) in MCF-7 cells and, the effects of Sp protein knockdown on ligand-dependent activation of pSp1₃ was investigated. Basal activity was decreased in cells transfected with iSp1, iSp3 and iSp4; however, fold induction by estrogens (E2 and DES), xenoestrogens and antiestrogens exhibited three patterns which differentially relied upon ER α /Sp1, ER α /Sp3, ER α /Sp4 or their combinations (Fig. 4–Fig. 6). For E2, HPTE, DES and HO-PCB-Cl₄, ER α /Sp1 > ER α /Sp4, and ER α /Sp3 had minimal to no effect on activation of ER α /Sp by these compounds. The pattern of ER α /Sp activation for BPA, endosulfan, NP and 4-OHT was ER α /Sp1 $\approx$ ER α /Sp4 with minimal contributions by ER α /Sp3. In contrast, both ER α /Sp1 and ER α /Sp3 play role in activation of pSp1₃ by ICI 182780 and kepone, but ER α /Sp4 tends to cause an inhibitory effect

since iSp4 enhances the fold induction by these compounds. These results demonstrate that for E2 and other ER agonists/antagonists, activation of the GC-rich pSp1₃ construct by ER α /Sp involves different combinations of ER α /Sp1, ER α /Sp3 and ER α /Sp4. Since E2-induced transactivation in MCF-7 cells transfected with pSp1₃ required ER α /Sp1 and ER α /Sp4, these results are in contrast with the requirement of ER α /Sp3 and ER α /Sp4 but not ER α /Sp1 for activation of the GC-rich VEGFR2 promoter by E2 [25]. This suggests that both cell- and promoter-context may also dictate which ER α /Sp complexes are required for ligand-induced transactivation. Therefore, the relative levels of Sp proteins expressed in a cell/tissue may also influence hormonal activation of specific genes, and this is currently being investigated using both cell culture and *in vivo* models.

In cells transfected with pSp1₃, xenoestrogens, estrogens and antiestrogens all activated ER α /Sp1. Therefore, xenoestrogen-induced interactions of ER α and Sp1 were investigated by FRET in MCF-7 cells transfected with CFP-Sp1 and YFP-ER α expression plasmids. This technique was previously used in this laboratory to show that E2 induced interactions of ER α and Sp1 in living cells [23]. We selected five compounds representative of estrogens, antiestrogens and xenoestrogens, and observed that E2, HO-PCB-Cl₄, kepone, BPA and 4-OHT all significantly induced FRET efficiencies (Figs. 7A and 7B). These data demonstrate a comparable pattern of ligand-dependent ER α -Sp1 interactions, and future studies will focus on ligand structure-dependent differences and similarities in the FRET efficiencies and kinetics of ER α interactions with Sp3 and Sp4.

CAD is induced by E2 in MCF-7 cells through ER α /Sp-dependent activation of proximal GC-rich promoter sequences [28;41]. Figure 7C illustrates that E2, HPTE and kepone induce CAD mRNA levels in MCF-7 cells, and these responses are abrogated in cells cotransfected with iSp1, iSp3 and iSp4 (combined). We have also observed similar results with CAD promoter constructs (data not shown) which have also been used for studies on xenoestrogens. In contrast, there are structure-dependent differences in activation of other putative ER α /Sp-dependent genes such as E2F1 and these are currently being investigated. These ongoing studies are also using E2-responsive genes that are repressed by E2 since this is a major pathway of estrogen action in breast cancer cells and other hormone-responsive cells and tissues.

In summary, results of this study show that xenoestrogens activate wild-type and variant ER α /Sp-dependent transactivation in MCF-7 cells transfected with pSp1₃. However, the results indicate that for some variant forms of ER α (i.e. ER α Δ ZF2/ER α Δ ZF1), most of the xenoestrogens resembled E2, whereas, in cells transfected with ER α (1–537), these compounds resembled the antiestrogens ICI 182,780 and 4-OHT. We also show for the first time that ligand structure-dependent activation of ER α /Sp in MCF-7 cells transfected with pSp1₃ was mediated by multiple Sp proteins (i.e. Sp1, Sp3 and Sp4) and utilization of these proteins was also ligand structure-dependent. There is evidence that Sp1 and Sp3 are differentially organized within the nucleus of MCF-7 cells [42], and this may contribute to the differences between ER α /Sp1 and ER α /Sp3 in activating pSp1₃ and we are also investigating the intranuclear distribution and organization of Sp1, Sp3 and Sp4. Results of this study, coupled with other reports of *in vitro* and *in vivo* assays with xenoestrogens [10–13;31–40], suggest that these compounds are SERMs and their ER α agonist and antagonist activities cannot necessarily be inferred from simple ER binding or transactivation assays.

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References

1. Nilsson S, Gustafsson JA. Biological role of estrogen and estrogen receptors. *Crit. Rev. Biochem. Mol. Biol* 2002;37:1–28. [PubMed: 11905545]
2. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J. Biol. Chem* 2001;276:36869–36872. [PubMed: 11459850]
3. Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS. Tripartite steroid hormone receptor pharmacology - interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Mol. Endocrinol* 1996;10:119–131. [PubMed: 8825552]
4. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selected receptor modulators. *Endocr. Rev* 2004;25:45–71. [PubMed: 14769827]
5. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J. Med. Chem* 2003;46:1081–1111. [PubMed: 12646017]
6. Jordan VC. Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 1. Receptor interactions. *J. Med. Chem* 2003;46:883–908. [PubMed: 12620065]
7. Krishnan V, Heath H, Bryant HU. Mechanism of action of estrogens and selective estrogen receptor modulators. *Vitam Horm* 2001;60:123–147. [PubMed: 11037623]
8. Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RG, Pike JW, McDonnell DP. Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol. Endocrinol* 1994;8:21–30. [PubMed: 8152428]
9. McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW. Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. *Mol. Endocrinol* 1995;9:659–669. [PubMed: 8592512]
10. Gould JC, Leonard LS, Maness SC, Wagner BL, Connor K, Zacharewski T, Safe S, McDonnell DP, Gaido KW. Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. *Mol. Cell. Endocrinol* 1998;142:203–214. [PubMed: 9783916]
11. Yoon K, Pallaroni L, Ramamoorthy K, Gaido K, Safe S. Ligand structure-dependent differences in activation of estrogen receptor α in human HepG2 liver and U2 osteogenic cancer cell lines. *Mol. Cell. Endocrinol* 2000;162:211–220. [PubMed: 10854714]
12. Yoon K, Pallaroni L, Stoner M, Gaido K, Safe S. Differential activation of wild-type and variant forms of estrogen receptor α by synthetic and natural estrogenic compounds using a promoter containing three tandem estrogen-responsive elements. *J. Steroid Biochem. Mol. Biol* 2001;78:25–32. [PubMed: 11530281]
13. Safe S, Papineni S. The role of xenoestrogenic compounds in the development of breast cancer. *Trends Pharmacol. Sci* 2006;27:447–454. [PubMed: 16822555]
14. Delfino F, Walker WH. Hormonal regulation of the NF- κ B signaling pathway. *Mol. Cell. Endocrinol* 1999;157:1–9. [PubMed: 10619392]
15. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. Estrogen receptor pathways to AP-1. *J. Steroid Biochem. Mol. Biol* 2000;74:311–317. [PubMed: 11162939]
16. Blobel GA, Orkin SH. Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. *Mol. Cell. Biol* 1996;16:1687–1694. [PubMed: 8657144]
17. Safe S, Kim K. Nuclear receptor-mediated transactivation through interaction with Sp proteins. *Prog. Nucleic Acid Res. Mol. Biol* 2004;77:1–36. [PubMed: 15196889]
18. Porter W, Saville B, Hoivik D, Safe S. Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol. Endocrinol* 1997;11:1569–1580. [PubMed: 9328340]
19. Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson J-A, Safe S. Ligand-, cell- and estrogen receptor subtype (α/β)-dependent activation at GC-rich (Sp1) promoter elements. *J. Biol. Chem* 2000;275:5379–5387. [PubMed: 10681512]
20. Kim K, Thu N, Saville B, Safe S. Domains of estrogen receptor α (ER α) required for ER α /Sp1-mediated activation of GC-rich promoters by estrogens and antiestrogens in breast cancer cells. *Mol. Endocrinol* 2003;17:804–817. [PubMed: 12576490]
21. Hutzinger O, Safe S, Zitko V. Preparation, gas chromatography behavior, and spectroscopic properties of hydroxylated chlorobiphenyls. *J. Assoc. Offic. Anal. Chem* 1974;57:1061–1067.

22. Gaido KW, Leonard LS, Maness SC, Galluzzo JM, McDonnell DP, Saville B, Safe S. Differential interaction of the methoxychlor metabolite HPTE with estrogen receptors alpha and beta. *Endocrinology* 1999;140:5746–5753. [PubMed: 10579340]
23. Kim K, Barhoumi R, Burghardt R, Safe S. Analysis of estrogen receptor alpha-Sp1 interactions in breast cancer cells by fluorescence resonance energy transfer. *Mol. Endocrinol* 2005;19:843–854. [PubMed: 15637147]
24. Sorkin A, McClure M, Huang F, Carter R. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol* 2000;10:1395–1398. [PubMed: 11084343]
25. Higgins KJ, Liu S, Abdelrahim M, Yoon K, Vanderlaag K, Porter W, Metz RP, Safe S. Vascular endothelial growth factor receptor-2 expression is induced by 17 β -estradiol in ZR-75 breast cancer cells by estrogen receptor α /Sp proteins. *Endocrinology* 2006;147:3285–3295. [PubMed: 16574784]
26. Stoner M, Wormke M, Saville B, Samudio I, Qin C, Abdelrahim M, Safe S. Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor α and Sp proteins. *Oncogene* 2004;23:1052–1063. [PubMed: 14647449]
27. Abdelrahim M, Samudio I, Smith R, Burghardt R, Safe S. Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells. *J. Biol. Chem* 2002;277:28815–28822. [PubMed: 12052832]
28. Khan S, Abdelrahim M, Samudio I, Safe S. Estrogen receptor/Sp1 complexes are required for induction of *cad* gene expression by 17 β -estradiol in breast cancer cells. *Endocrinology* 2003;144:2325–2335. [PubMed: 12746293]
29. Watson CS, Campbell CH, Gametchu B. The dynamic and elusive membrane estrogen receptor- α . *Steroids* 2002;67:429–437. [PubMed: 11960618]
30. Levin ER. Integration of the extranuclear and nuclear actions of estrogen. *Mol. Endocrinol* 2005;19:1951–1959. [PubMed: 15705661]
31. Jordan VC, Schafer JM, Levenson AS, Liu H, Pease KM, Simons LA, Zapf JW. Molecular classification of estrogens. *Cancer Res* 2001;61:6619–6623. [PubMed: 11559523]
32. An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. Estrogen receptor β -selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J. Biol. Chem* 2001;276:17808–17814. [PubMed: 11279159]
33. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, Nilsson S. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. *Mol. Pharmacol* 1998;54:105–112. [PubMed: 9658195]
34. Mueller SO, Simon S, Chae K, Metzler M, Korach KS. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor α (ER α) and ER β in human cells. *Toxicol. Sci* 2004;80:14–25. [PubMed: 15084758]
35. Hall JM, McDonnell DP, Korach KS. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Mol. Endocrinol* 2002;16:469–486. [PubMed: 11875105]
36. Bentrem D, Fox JE, Pearce ST, Liu H, Pappas S, Kupfer D, Zapf JW, Jordan VC. Distinct molecular conformations of the estrogen receptor α complex exploited by environmental estrogens. *Cancer Res* 2003;63:7490–7496. [PubMed: 14612550]
37. Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol* 1999;154:76–83. [PubMed: 9882594]
38. Matthews JB, Fertuck KC, Celius T, Huang YW, Fong CJ, Zacharewski TR. Ability of structurally diverse natural products and synthetic chemicals to induce gene expression mediated by estrogen receptors from various species. *J. Steroid Biochem. Mol. Biol* 2002;82:181–194. [PubMed: 12477484]
39. Routledge EJ, White R, Parker MG, Sumpter JP. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) α and ER α . *J. Biol. Chem* 2000;275:35986–35993. [PubMed: 10964929]
40. Fujita T, Kobayashi Y, Wada O, Tateishi Y, Kitada L, Yamamoto Y, Takashima H, Murayama A, Yano T, Baba T, Kato S, Kawabe Y, Yanagisawa J. Full activation of estrogen receptor alpha

- activation function-1 induces proliferation of breast cancer cells. *J. Biol. Chem* 2003;278:26704–26714. [PubMed: 12738788]
41. Khan S, Wu F, Liu S, Wu Q, Safe S. Role of specificity protein (Sp) transcription factors in estrogen-induced gene expression in MCF-7 breast cancer cells. *J. Mol. Endocrinol.* 2007(in press)
42. He S, Sun JM, Li L, Davie JR. Differential intranuclear organization of transcription factors Sp1 and Sp3. *Mol. Biol. Cell* 2005;16:4073–4083. [PubMed: 15987735]

Figure 1.

Activation of ER α /Sp by estrogens, antiestrogens, xenoestrogens and resveratrol in MCF-7 cells. Induction by E2/DES (A) and 4-OHT/ICI 182,780 (B). MCF-7 cells were transfected with pSp1₃ and ER α expression plasmid, treated with different concentrations of E2, DES, 4-OHT or ICI 182,780, and luciferase activity determined as described in the Materials and Methods. Each point is the mean $\pm$ SD for three replicate determinations and significant ($p < 0.05$) induction is indicated (*). E2 does not induce luciferase activity in MCF-7 cells transfected with pSp1₃ alone and E2-responsiveness requires cotransfection with ER α . Induction by HPTE/BPA (C), octylphenol/nonylphenol (D), HO-PCB-Cl₄/resveratrol (E), kepon/endosulfan (F). MCF-7 cells were transfected with pSp1₃ and ER α , treated with

different concentrations of the various ER α agonists and luciferase activity determined as described in the Materials and Methods. Each point is the mean $\pm$ SD for three replicate determinations and significant ($p < 0.05$) induction is indicated (*).

Figure 2.

Structure-dependent activation of variant ER α /Sp in MCF-7 cells/Transfection with wild-type ER α (A), ER α Δ ZF1 (B), ER α Δ ZF2 (C), ER α (1-553) (D), and ER α (1-537) (E). Cells were transfected with pSp1₃ and variant forms of ER α , treated with ER α agonists and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SD for three replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*). The concentrations used in this experiment were those which maximally induced luciferase activity using pSp1₃ and wild-type ER α (Fig. 1 and Fig. 2) without causing any cytotoxicity.

Figure 3.

Structure-dependent activation of wild-type and variant $ER\alpha/Sp$ in MDA-MB-231 cells. Transfection with wild-type $ER\alpha$ (A), $ER\alpha\Delta ZF1$ (B), $ER\alpha\Delta ZF2$ (C), $ER\alpha(1-553)$ (D), and $ER\alpha(1-537)$ (E). MDA-MB-231 cells were transfected with pSp13, wild-type or variant $ER\alpha$, treated with ER agonists and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SD for three replicate determinations for each treatment group and significant ($p < 0.05$) induction is indicated (*). Preliminary concentration-dependent studies were carried out in cells transfected with pSp13 and wild-type $ER\alpha$, and the concentrations of each compound that maximally-induced activity without cytotoxicity were used in all experiments.

Figure 4.

RNA interference studies with iSp1, iSp3 and iSp4. (A) Western immunoblot analysis. MCF-7 cells were transfected with iSp1, iSp3, iSp4, iNS (non-specific), or various combinations, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Quantitation of Sp knockdown was determined using three replicates for each treatment and levels were normalized to β-actin. Significantly ($p < 0.05$) decreased levels are indicated (*) and results are compared to those observed in the DMSO group (set at 1.0). Effects of Sp protein knockdown on activation of pSp13 by E2 (B), 4-OHT (C), and HPTE (D). MCF-7 cells were transfected with pSp13, ERα and small inhibitory RNAs for iNS or Sp proteins and treated with 10 nM E2, 1 μM 4-OHT, or 25 μM HPTE. Luciferase activity was determined as

described in the Materials and Methods, and results are means $\pm$ SD for at least three replications for each treatment group. Significantly ($p < 0.05$) induced activity relative to DMSO is indicated (*) and a decrease in induced activity (compared to iNS) by iSp1, iSp3, iSp4 or their combinations is indicated by a double asterisk (**).

Figure 5.

Differential role of Sp proteins in activation of ER α /Sp by ER α agonists. Effects of Sp protein knockdown on activation of pSp1₃ by DES (A), HO-PCB-Cl₄ (B), BPA (C), nonylphenol (D), and endosulfan (E). MCF-7 cells were transfected with pSp1₃/ER α and small inhibitory RNAs, treated with various ER α ligands, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SD for three replicate determinations for each treatment group. Significantly ($p < 0.05$) induced activity relative to DMSO is indicated (*) and a decrease in induced activity (compared to iNS) by iSp1, iSp3 or iSp4 is indicated by a double asterisk (**).

Figure 6.

Differential role of Sp proteins in activation of ER α /Sp by ICI 182,780 (A and B) or kepone (C and D). MCF-7 cells were transfected with pSp1₃/ER α and various iSp_s, treated with 1 μ M ICI 182,780 or 5 μ M kepone, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means $\pm$ SD for three replicate determinations for each treatment group. Significantly ($p < 0.05$) induced activity relative to DMSO is indicated (*) and a decrease in induced activity (compared to iNS) by iSp1, iSp3 or iSp4 is indicated by a double asterisk (**).

Figure 7.

FRET analysis and induction of E2F1 in MCF-7 cells. (A) An example of FRET in MCF-7 cells treated with DMSO (upper panel) and 10 μ M kepone (lower panel) for 20 – 30 min. MCF-7 cells were transfected with CFP-Sp1 and YFP-ER α , and each panel represents the pseudocolor intensity of CFP, YFP and FRET signals, respectively. (B) FRET efficiencies. MCF-7 cells were transfected with CFP-Sp1 and YFP-ER α , treated with DMSO, 10 nM E2, 20 μ M HO-PCB-Cl₄, 10 μ M kepone, 75 μ M BPA and 1 μ M 4-OHT for 20 – 30 min, and FRET efficiencies were determined as described in the Materials and Methods. Significant differences ($p < 0.05$) in treated cells from DMSO (control) are indicated (*). (C) Role of Sp proteins in activation of CAD mRNA. MCF-7 cells were transfected with non-specific RNA (iNS) or iSp1,

iSp3 and iSp4 (combined), treated with DMSO or 10 nM E2, 5 μ M kepone, or 20 μ M HPTE for 6 hr, and CAD mRNA levels were analyzed by real time-PCR as described in the Materials and Methods. Experiments were carried out in triplicate. Results are expressed as means $\pm$ SD and the CAD mRNA levels (normalized to TBP mRNA) in the DMSO treatment group was set at 1.0. Significant ($p < 0.05$) induction is indicated by an asterisk, and significant inhibition after transfection with iSp1, iSp3 and iSp4 (combined; 25 nM for each construct) is also indicated (**).