Published in final edited form as:

Mol Cell Endocrinol. 2016 December 05; 437: 190–200. doi:10.1016/j.mce.2016.08.025.

Estrogen receptor- α and aryl hydrocarbon receptor involvement in the actions of botanical estrogens in target cells

Ping Gong^a, Zeynep Madak-Erdogan^a, Jodi A. Flaws^b, David J. Shapiro^c, John A. Katzenellenbogen^d, and Benita S. Katzenellenbogen^{a,*}

^aDepartment of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^bDepartment of Comparative Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^cDepartment of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^dDepartment of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Abstract

Botanical estrogen (BE) dietary supplements are consumed by women as substitutes for loss of endogenous estrogens at menopause. To examine the roles of estrogen receptor α (ER α) and aryl hydrocarbon receptor (AhR) and their crosstalk in the actions of BEs, we studied gene regulation and proliferation responses to four widely used BEs, genistein, daidzein, and S-equol from soy, and liquiritigen from licorice root in breast cancer and liver cells. BEs and estradiol (E2), acting through ER α , stimulated proliferation, ER α chromatin binding and target-gene expression. BEs but not E2, acting through AhR, bound to xenobiotic response element-containing chromatin sites and enhanced AhR target-gene expression (CYP1A1, CYP1B1). While E2 and TCDD acted quite selectively through their respective receptors, BEs acted via both receptors, with their AhR activity moderated by negative crosstalk through ER α . Both ER α and AhR should be considered as mediators of the biology and pharmacology of BEs.

Keywords

Estrogen receptor; Aryl hydrocarbon receptor; Gene regulation; Botanical estrogens; Cell proliferation; Xenobiotic metabolism

1. Introduction

Estrogens are global regulators of human physiology, and they have many beneficial effects on diverse non-reproductive target tissues in postmenopausal women, but they can also stimulate proliferation of reproductive tissues, driving pathological processes such as breast and uterine cancers. Botanical estrogens (BEs), which are estrogenic substances obtained

*Corresponding author. katzenel@illinois.edu (B.S. Katzenellenbogen).

Disclosure statement

The authors have nothing to disclose.

from a variety of plants, are present in many dietary supplements widely consumed by women, especially older women, in the hope of preserving their health and preventing undesired changes that result from the sharp decline in estrogen levels at menopause. These include loss of bone mass, increased weight gain and adiposity, glucose intolerance, and diminished vascular health (Santen et al., 2010; Simpson and Santen, 2015). These health challenges are accentuated because women now live many years after menopause. Many users of these BEs take them believing that these compounds will provide a safe, natural alternative to pharmaceutical hormone replacement therapy.

BEs were so named because these naturally occurring compounds in plants have been shown to bind to estrogen receptors (ERs) and to have the ability to stimulate the expression of estrogen target genes and to elicit phenotypic changes in estrogen target tissues and cells that are in some cases similar to those of the endogenous estrogen, estradiol (E2) (Boonmuen et al., 2016; Jiang et al., 2013). However, our profiling of genome-wide transcriptional response to different BEs and to E2 revealed that not all BEs exhibited the same pattern of gene regulations and that they also showed some effects on gene expression quite different from that of E2 (Gong et al., 2014).

The ER α is able to bind a variety of structurally diverse ligands, both naturally occurring and synthetic. Some of these ligands, such as the selective estrogen receptor modulators (SERMs), induce distinct conformational changes in ER α that generate a spectrum of transcriptional complexes exhibiting tissue- and cell-selective agonist and antagonist activities (Frasor et al., 2004; Maximov et al., 2013; McDonnell and Wardell, 2010; Wardell et al., 2012). Recently, studies by us and others have documented crosstalk of ER α with other intracellular receptors, such as the aryl hydrocarbon receptor (AhR) (DuSell et al., 2010; Madak-Erdogan and Katzenellenbogen, 2012). Consistent with the major role of AhR in upregulating the expression of genes for xenobiotic metabolizing enzymes, the AhR is activated by a variety of structurally diverse exogenous chemicals and endogenous compounds (Denison and Nagy, 2003; Denison et al., 2011). Based on the promiscuity of AhR activation by ligands having diverse structures and evidence that ligands for ERs might also interact with receptor targets other than ER, we have examined herein the roles of ER α and AhR in mediating the ability of estrogenic compounds from plants to modulate the transcriptional activity of both ER α and AhR. We also have examined how crosstalk between these two receptors might affect gene regulation and alter the phenotypic properties (such as the metabolism and proliferation) of breast cancer cells and hepatocytes, two cell types that contain both ER α and AhR, and thus are potential targets for the actions of BEs. Our findings provide evidence that the actions of BEs on gene regulation and the behavior of these target cells are mediated through both ER α and AhR, implying that both receptors need to be considered as regulators of the biology and pharmacology of BEs. Notably as well, the actions of BEs on AhR differ from that of E2.

2. Materials and methods

2.1. Ligands and BE compounds

17 β -Estradiol (E2), dioxin (TCDD), and ICI 182,780 were from Sigma-Aldrich. CH 223191 was from Santa Cruz Biotech. The sources of genistein, daidzein, S-equol, and liquiritigenin

were as described previously (Jiang et al., 2013). All compounds were dissolved in ethanol prior to addition to cell culture media at 1:1000 dilutions.

2.2. Human breast cancer and hepatoma cell cultures

MCF-7 human breast cancer cells and HepG2 hepatoma cells stably containing ER α (HepG2-ER α cells) (Kanamori et al., 2000; Krieg et al., 2004) were maintained as previously described (Jiang et al., 2013). Six days prior to treatment with compounds, cells were seeded in 6-well plates using phenol red-free media supplemented with 5% charcoal dextran-treated calf serum. Medium was changed on days 2 and 4, and cells were treated with compounds beginning on day 6.

2.3. siRNA transfection of cells

Short interfering RNA (siRNA) knockdown of ER α and AhR was performed as described previously (Madak-Erdogan and Katzenellenbogen, 2012). Briefly, cells were seeded in 6-well plates using phenol red-free media at a density of 2.5×10^5 cells/well and transfected the next day with 25 nM control siRNA or 25 nM ER α siRNA or AhR siRNA using the DharmaFECT1 Transfection Reagent (GE Healthcare, Cat# T-2001-03). After 48 h, the cells were treated with compounds for the times indicated. siGE-NOME ESR1 siRNA SMART pool (Cat# M-003401-04) and ON-TARGET plus human AHR(196) siRNA SMART pool (Cat# L-004990-00) were obtained from GE Healthcare. The efficacies of siRNA knockdown of ER α and AhR were verified by qPCR and Western blot. Total cell lysate protein was subjected to Western blot analysis with anti-ER α antibody F-10 (Santa Cruz, sc-8002) and anti-AhR antibody H-211 (Santa Cruz, sc-5579).

2.4. Mice and the isolation, culture and treatment of mouse primary hepatocytes

All protocols using animals were approved by the University of Illinois Animal Care and Use Committee. Isolation of mouse primary hepatocytes from AhR knockout female mice and their wildtype female littermates was modified from the procedure described (Hernandez-Ochoa et al., 2013; Schmidt et al., 1996). Six week-old female mice were sacrificed by CO₂ inhalation, perfused transcardially first with 20 ml of Perfusion Buffer I [Hank's Balanced Salt Solution (HBSS) without Ca²⁺ and Mg²⁺ (Invitrogen, Cat# 14174) supplemented with 10 mM HEPES, 0.075% sodium bicarbonate, and 1.2 mM EDTA (pH 8.0)] and then with 20 ml of Perfusion Buffer II [HBSS with Ca²⁺ and Mg²⁺ (Invitrogen, Cat# 14025) supplemented with 25 mM HEPES and 3 unit/ml collagenase II (Worthington)] through the hepatic portal vein. After perfusion, the liver was placed in cold William's Complete Medium [William's Medium E (Invitrogen, Cat# 12551-032) supplemented with 2 mM L-glutamine, 5% fetal bovine serum, 100 nM insulin, 100 nM dexamethasone, 100 units/ml penicillin and 100 mg/ml streptomycin]. Hepatic cells were dispersed from the mouse liver and washed twice with cold William's Complete Medium, resuspended in warm William's Complete Medium, and seeded into 6-well dishes at a density of 5×10^5 cells/well. Media were changed to William's Complete Medium plus 1% DMSO after 4 h to retain cell surface receptors. After 16 h, the cells were treated with compounds for the times indicated.

2.5. RNA isolation and real-time PCR

Total RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using MMTV reverse transcriptase (New England Bio-Labs). Real-time PCR was performed using SYBRgreen PCR Master Mix Roche as described (Madak-Erdogan and Katzenellenbogen, 2012). The primers used for the genes monitored in this study are listed in Table 1.

2.6. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described (Madak-Erdogan et al., 2016a; Madak-Erdogan and Katzenellenbogen, 2012). MCF-7 cells were treated with 0.1% ethanol or 10 nM E2 or 1 nM TCDD or 1 μ M Liquiritigenin or S-equol for 45 min, fixed with 0.8% of formaldehyde for 15 min, and lysed with lysis buffer [10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1% SDS and 0.5% Empigen], supplemented with a protease and phosphatase inhibitor cocktail (Roche) just before use. The cell lysates containing cross-linked chromatin complexes were sonicated and immunoprecipitated with ER α antibody HC-20 (Santa Cruz, sc-543) for ER α -DNA complex or AhR antibody H-211 (Santa Cruz, sc-5579) for AhR-DNA complexes. The ChIP DNA was used for quantitative real-time PCR. Primers used to amplify ER α or AhR binding regions to genes of interest are listed in Table 1. Primers to a known non-ER α or AhR binding region of the pS2 gene were used as a negative control.

2.7. Cell proliferation assays

WST-1 assay (Roche, Basel, Switzerland) was used to quantify cell viability as described previously (Jiang et al., 2013). Absorbance was measured at 450 nm using a VICTOR X5 PerkinElmer 2030 Multilabel Plate Reader, and all assays were performed in triplicate.

2.8. Statistical analyses

All data were analyzed by Student's *t*-test or one-way ANOVA with Dunnett's multiple comparison test, as appropriate. Findings were considered significant if *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. Botanical estrogens (BEs) and E2 stimulate the expression of ER α target genes, whereas BEs and TCDD activate and E2 represses the expression of CYP1A1 and CYP1B1 genes in breast cancer cells

When MCF-7 cells were treated with E2 or BEs, the expression of typical ER α target genes, such as the progesterone receptor (PgR) and pS2, were up-regulated by E2 and BEs, but were unaffected by the AhR ligand TCDD/dioxin (Fig. 1A). While the AhR target genes CYP1A1 and CYP1B1 were up-regulated by the AhR agonist TCDD, they were down-regulated by E2; however, they were stimulated, not inhibited by the BEs (Fig. 1B). This pattern of gene regulation by the different compounds, shown at 4 h of treatment with compounds in Fig. 1, was also observed at 24 h of treatment. The magnitude of expression of the ER target genes was similar at 4 h and 24 h, whereas expression of the CYP1A1 and CYP1B1 genes was slightly (1.5-fold) greater at 24 h than at 4 h.

Fig. 2 shows dose-response relationships in the regulation of the ER α target gene, PgR, and the AhR target gene, CYP1A1. Low concentrations of E2 (10^{-11} – 10^{-9} M) almost fully down-regulated expression of the CYP1A1 gene and greatly up-regulated expression of the PgR gene. TCDD stimulated CYP1A1 gene expression at 10^{-9} – 10^{-8} M concentration but did not stimulate PgR gene expression over the broad concentration range tested (Fig. 2). Of note, higher concentrations of BEs (10^{-7} – 10^{-5} M) were needed to elicit regulation of PgR and CYP1A1 gene expression compared with E2 or TCDD, and approximately 10-fold higher concentrations of the BEs were required for stimulation of the AhR vs. the ER α target gene (Fig. 2). Of the BEs, genistein increased PgR expression at 10^{-8} M, about 10-fold lower than that of the other BEs (Fig. 2), likely reflecting its higher affinity for ER α (Jiang et al., 2013). EC₅₀ values for these responses are given in Table 2.

3.2. Activation of ER α and AhR target genes by BEs and crosstalk between ER α and AhR

To examine the involvement of ER α and/or AhR in the actions of BEs in regulating ER α and AhR target genes, we blocked ER α or AhR function using the ER α antagonist ICI 182,780 (ICI) or the AhR antagonist CH 223191 (CH), and we also depleted cells of ER α or AhR by knockdown of ER α or AhR using specific siRNAs. As seen in Fig. 3A, treatment with the antiestrogen ICI fully blocked stimulation of PgR and pS2, ER α target genes, by E2 and BEs, whereas the AhR antagonist CH was without effect. By contrast, ICI reversed E2 suppression of the AhR target genes CYP1A1 and CYP1B1 and increased basal expression of these genes, but had little or no effect on TCDD and BE stimulation of expression of these genes. Treatment with the AhR antagonist CH effectively prevented regulation of CYP1A1 and CYP1B1 by all ligands tested (Fig. 3A).

These findings with ER α or AhR inhibitors were supported by observations made when cells were depleted of ER α or AhR using receptor-specific siRNA. As seen in Fig. 3B, use of siER α but not siAhR eliminated the response of PgR and pS2 to all ligands. By contrast, the regulation of CYP1A1 or CYP1B1 by ligands was eliminated by knockdown of AhR but not knockdown of ER α (Fig. 3B). The Western blot inset in Fig. 3B, lowest right panel, shows that the siRNAs were selective for ER α or AhR and were effective in reducing the cellular protein levels of each receptor by over 90%. The results clearly show that BEs were able to activate CYP1A1 and CYP1B1 in the absence of functional ER α (Fig. 3B). However, it is of note that when ER α was inhibited with ICI (Fig. 3A) or depleted from cells using siRNA (Fig. 3B), we obtained a greater response of the AhR target genes to E2 and to the BEs. This was more obvious with siRNA knockdown of ER α (Fig. 3B), where basal expression of CYP1A1 and CYP1B1 was increased and the BEs elicited a much greater gene stimulation. The data support crosstalk between ER α and AhR, with ER α exerting a negative and suppressive effect on AhR target genes.

Because cell context and tissue type can affect gene response to ligands, we next assessed BE actions in primary hepatocytes from mouse liver, a cell in which AhR carries out major functions including gene regulation (Hernandez-Ochoa et al., 2013; Schmidt et al., 1996). When we treated primary hepatocytes isolated from wild type or AhR knockout mice with BEs, TCDD, or E2, we observed a nearly complete loss of the activation of CYP1A1, CYP1B1, and leptin by BEs and TCDD in hepatocytes from AhR knockout mice compared

to what was seen in the hepatocytes of WT mice (Fig. 4A,B). TCDD and related polychlorinated biphenyls have been shown to stimulate expression of leptin (Ferrante et al., 2014; Linden et al., 2014; Wahlang et al., 2013). These results indicate that, as observed in breast cancer cells, BEs also activate AhR target genes in hepatocytes through AhR. Of note, E2 elicited only limited stimulation of expression of these three genes, and E2 did not show the down-regulation of CYP1A1 or CYP1B1 gene expression in mouse primary hepatocytes that was observed in MCF-7 cells (Fig. 3B). In primary mouse hepatocytes, E2, TCDD and BEs also up-regulated leptin, a satiety hormone involved in regulation of lipid metabolism and this stimulation was not obtained in hepatocytes from AhR knockout mice (Fig. 4C). In HepG2 hepatoma cells stably expressing ER α (Fig. 4D,E), the ER α target gene and granzyme inhibitor, proteinase inhibitor-9 (PI9) was up-regulated only by ethinyl estradiol (EE2) and by the BEs and not by the AhR agonist TCDD, and this up-regulation was blocked by ICI. By contrast, the AhR target gene CYP1A1 was up-regulated by TCDD and the BEs, and this was prevented by cotreatment with CH223191. As observed in MCF-7 cells, cotreatment with the antiestrogen and ER α degrader, ICI, augmented the magnitude of gene up-regulation by E2 and the four BEs (Fig. 4E). Notably, the basal vehicle level of CYP1A1 gene expression was also increased, as observed also in MCF-7 cells shown in Fig. 3, suggesting release from a suppressive effect of ER α on AhR regulated gene control when cells are treated with the antiestrogen, ICI, or when cells are depleted of ER α by siRNA knockdown.

3.3. Effects of botanical estrogens on cell proliferation

To look beyond gene regulation, we examined the impact of BE ligands on cell proliferation, monitoring expression of the proliferation marker Ki67 (Fig. 5A) and the growth rate and numbers of MCF-7 cells monitored after 6 days (Fig. 5B,C). E2 and the BEs increased cell proliferation and Ki67 mRNA level, both of which were fully reversed by the antiestrogen ICI but were minimally altered, if at all, by the AhR inhibitor CH. These findings imply that proliferation is regulated by ER α . In keeping with this, the AhR ligand TCDD did not change proliferation or Ki67 gene expression in these cells (Fig. 5A and C), even over a broad concentration range tested (Fig. 5B), whereas low concentrations of E2 were able to increase cell proliferation (Fig. 5B).

3.4. Examination of the recruitment of ER α and AhR by BEs to chromatin binding sites in ER α and AhR target genes

To investigate the mechanism underlying the ability of BEs to regulate both ER α target genes and AhR target genes, we examined the recruitment of ER α and AhR to estrogen-responsive regions of ER α target genes and to AhR-responsive regions in the CYP1A1 and CYP1B1 genes. As seen in Fig. 6A, treatment with E2 and the BEs, but not TCDD, induced the recruitment of ER α to classical estrogen binding sites in the estrogen target genes, TFF1/pS2 and LRRC54. As expected, AhR was minimally or not at all recruited to these estrogen-responsive regions by any of the ligands (Fig. 6A). At the known xenobiotic response element (XRE) region in the CYP1A1 promoter (at -1 Kb) and in the CYP1B1 promoter (at -0.8 Kb upstream from the transcription start site), TCDD and the BEs, but not E2, induced greatly increased AhR recruitment (Fig. 6B). E2 and BEs elicited only a weak enhancement of ER α binding to the XRE. Thus, BEs bound to ERE-containing regions in

estrogen target genes via ER α and also bound to XRE-containing regions in AhR target genes largely via AhR and regulated expression of these genes, whereas E2 and TCDD showed more highly selective ER α -ERE or AhR-XRE chromatin binding and gene regulation.

3.5. Effects of TCDD and BE cotreatment on the expression of ER α and AhR target genes

As shown in Fig. 7, we examined the impact of cotreatment with TCDD along with E2 or each of the 4 BEs on the expression of ER α or AhR target genes. The magnitude of activation of ER α target genes (PgR and pS2) by E2 or BEs was not affected by cotreatment with TCDD, and TCDD treatment alone did not stimulate expression of these ER target genes (Fig. 7A). By contrast, for the AhR target gene CYP1A1 (Fig. 7B), TCDD elicited robust up-regulation of expression of this gene and E2, and each of the 4 BEs significantly suppressed the stimulation by TCDD, indicating that these 5 estrogens act as mixed agonists/antagonists on this gene. By contrast, TCDD and E2 and the 4 BEs alone elicited different levels of CYP1B1 gene stimulation, and E2 and BEs did not significantly change the magnitude of CYP1B1 gene stimulation elicited by TCDD (Fig. 7B). Thus, E2 and the 4 BEs showed different agonist and/or antagonist activities on these two genes, consistent with previously reported AhR-mediated agonistic/antagonistic activities of food flavonoids using human and rat reporter gene assays (Van der Heiden et al., 2009).

4. Discussion

Botanical estrogens in dietary supplements are widely used by postmenopausal women because they are viewed as being a natural and safe alternative to pharmaceutical estrogen replacement therapies. In this study, we compared the activities of three compounds from soy, namely genistein, S-equol and daidzein, and a major estrogenic component from licorice root, liquiritigenin, with each other and with estradiol (E2). Notably, these four BEs all behaved similarly to one another in up regulating both ER α and AhR target genes in human breast cancer cells and in primary hepatocytes and in HepG2-ER α hepatoma cells, and in enhancing the proliferation of breast cancer cells. By contrast, E2 up regulated ER α target genes and proliferation of breast cancer cells, and suppressed AhR target gene expression in breast cancer cells; E2 also elicited little if any stimulation of AhR target genes, which were more greatly stimulated by BEs and by TCDD in the two types of liver cells examined. These findings were supported by differences observed in the chromatin recruitment of ER α and AhR by BEs versus E2 and TCDD to ER α and AhR target genes. Others have previously shown agonistic effects of selected isoflavones on AhR activities in reporter gene cell line assays (Bialesova et al., 2015; Van der Heiden et al., 2009). It is notable as well that the direction and magnitude of gene regulations by E2 were very cell-specific and differed in breast vs. liver target cells, likely reflecting differences in the cell background of coregulators and other cofactors that work along with ER α and AhR in different cells (McKenna et al., 2014; Stender et al., 2010). Also, cotreatment studies with TCDD and E2 and each of the 4 BEs revealed the AhR gene-selective agonistic and antagonistic actions of the 5 estrogens, supporting previous work that showed AhR-mediated agonistic/antagonistic activities of food flavonoids using reporter gene assays (Van der Heiden et al., 2009). Thus, our findings highlight that BEs work through both the ER α and

the AhR to regulate gene expression, but that BEs and E2 may have different effects on xenobiotic metabolism regulated by these cytochrome P450 enzymes, at least in some cells.

An important issue is how the concentrations of botanical estrogens used in our *in vitro* cell studies relate to those found in the blood of women consuming botanical estrogens in dietary supplements. There are many uncertainties and assumptions in these determinations, because both soy and licorice can be taken as foods, powders, and as dietary supplements containing concentrated soy and licorice components, the levels of which can vary markedly (Setchell et al., 2001), and in the case of licorice may be from the roots of different *Glycyrrhiza* species.

Women consuming soy-based foods generally have 0.2–0.5 μM blood levels of total soy isoflavones (genistein and daidzein, and their conjugates) (Morton et al., 1994), with considerable inter-individual variability (Xu et al., 1995), but blood levels after consumption of high-dose dietary supplements can be considerably higher, 0.75–6.0 μM (Cassidy et al., 2006a, 2006b; Doerge et al., 2000; Jiang et al., 2013). With aglycone levels being ca. 2% of the total (Setchell et al., 2001), this can give blood levels of the free isoflavones of 0.015–0.12 μM , which are clearly in the range at which we see that these soy BEs have activity on ER-regulated genes, and within an order of magnitude at which they stimulate AhR-regulated genes in cells. Human exposure to S-equol is largely due to reductive enteric metabolism of daidzein in soy dietary supplements. This conversion can vary greatly depending on the composition of the individual's gut microbiota (Cassidy et al., 2006b), and hence is more difficult to quantify. Total Liq is estimated to be approximately 4 μM in the serum of women taking licorice root supplements, with the aglycone form being ca. 1% of the total Liq in the serum (0.04 μM) (Madak-Erdogan et al., 2016b), although peak levels in serum could well be higher soon after supplement consumption. We see some stimulation of ER-regulated genes by Liq at 0.1 μM , but higher concentrations are required for good stimulation of AhR-regulated genes.

Our observations support prior reports by us and others (Ahmed et al., 2009; Beischlag and Perdew, 2005; Denison and Nagy, 2003; DuSell et al., 2010; Madak-Erdogan and Katzenellenbogen, 2012; O'Donnell et al., 2014) documenting crosstalk between ER α and AhR by estradiol and by the SERMs tamoxifen and raloxifene, and we extend these now to include BEs from soy and licorice root. We and others have reported extensive interrelationships between ER α and AhR complexes in regulation of target genes and cell proliferation of breast cancer cells (Liu et al., 2006; Madak-Erdogan and Katzenellenbogen, 2012; Tiong et al., 2012). In addition, we found that in breast cancer cells, the expression of AhR target genes was decreased by E2 whereas here we found that soy BEs and liquiritigenin stimulated AhR target gene expression, as did the potent AhR agonist TCDD/dioxin. Thus, different ER α ligands can have opposite effects on AhR activity, implying that in these cells these ligands might have potentially differing impacts on the metabolism of drugs and polycyclic aromatic hydrocarbons by these AhR-regulated enzymes. Furthermore, the AhR stimulatory actions of these BEs were blocked by the AhR inhibitor, CH233191, but not by ICI, implying that their actions on CYP1A1 and 1B1 and leptin, are mediated by AhR and not ER α . By contrast, E2 and all four BEs (but not TCDD) activated ER α -

responsive genes (e.g., PgR, pS2), with BE stimulation of these genes being blocked by ICI but not by CH233191.

Because some of the ligands that regulate the activity of AhR also activate the xenobiotic-sensing receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Timsit and Negishi, 2007), we used liver from AhR knockout mice and also used the AhR antagonist CH233191 in cell cultures to confirm that the regulation of the cytochrome P450 and leptin genes by BEs was indeed mediated by AhR. It is of note that in primary mouse hepatocytes, where we examined the stimulation of leptin, we found that E2, TCDD, genistein, daidzein, S-equol and liquiritigenin all increased leptin expression, with stimulation being lost in hepatocytes from AhR knockout mice. The four BEs consistently up-regulated expression of AhR target genes in the different cell types examined (breast cancer cells, primary hepatocytes and human hepatoma cells) and this up regulation by the BEs required AhR. The direction of CYP1A1 and CYP1B1 gene expression regulation by E2 depended on cell context and differed in MCF-7 breast cancer cells versus liver cells. This indicates that gene regulation by different estrogens through AhR is cell-specific and could contribute to tissue-selective effects of BEs *in vivo*. Furthermore, the flavonoid BEs can undergo rapid metabolism in cultured cells and in tissues *in vivo* (Spencer et al., 2004), and this metabolism could contribute in their cell-specific and tissue-specific effects. Thus, BEs appear to function much like selective estrogen receptor modulators (SERMs) in that they have cell-selective estradiol-like activities through ER α but also exhibit cell-selective dioxin-like activities through AhR. The cell-context dependence of gene regulation by nuclear receptors is well documented and is thought to be determined by differences in cell background, in particular by differences in the contents of coregulators and other cofactors with which these receptors bind to form multicomponent complexes that regulate gene expression at the chromatin level (Lonard and O'Malley, 2012; McKenna et al., 2014; Smith and O'Malley, 2004; Stender et al., 2010). In fact, it is noteworthy that ER α and AhR interact with many of the same coregulators and cofactors, including the coregulators RIP140 (Madak-Erdogan and Katzenellenbogen, 2012), SRC 1, 2, and 3 (Endler et al., 2012; Madak-Erdogan and Katzenellenbogen, 2012) and FOXA1 (Ahmed et al., 2012).

The network of activation, inhibition and crosstalk of the ER α and AhR regulatory systems as modulated by E2, TCDD, and BEs is complex, and is schematized in the model presented in Fig. 8. The actions of E2 and TCDD are quite specific for activation of their cognate receptor targets, ER α and AhR, respectively. By contrast, the BEs examined have dual activities, activating ER α target genes through ER α and regulating AhR target genes through AhR. At the same time, E2 and the BEs can moderate AhR activity through negative crosstalk mediated by ER α ; however, they do so in somewhat different ways. The activation of AhR by BEs can be moderated indirectly by negative crosstalk through their action on ER α ; by contrast, while E2 itself does not activate AhR, by using the same negative crosstalk through ER α , it can moderate AhR activity stimulated by other ligands such as TCDD. It is not clear whether the suppressive effect of the estrogens acting through ER on the expression of AhR target genes is due to a direct competitive binding effect between ER and AhR at the chromatin level (Fig. 6B) or to more indirect effects through ER, such as those involving competition for shared coregulators, noted above. In terms of cell proliferation, this appeared to be stimulated by E2 and BE action through ER α only; this

enhanced proliferation was fully suppressed by ICI, and was essentially unaffected by TCDD and by the AhR antagonist, CH223191. As others have shown, the effects of these compounds on the proliferative, invasive, and migratory activities of MCF-7 cells are not mediated through AhR (Spink et al., 2013). None-the-less, there is considerable current interest in AhR as a possible prognostic marker in breast cancer, since the presence of AhR is associated with better overall patient survival (Saito et al., 2014).

5. Conclusion

The studies reported here provide new insights into the actions of several BEs from soy and licorice root, mediated through ER α and/or AhR, on the regulation of ER α and AhR target genes, and their impact on cell proliferation and potential xenobiotic metabolism. The BEs exerted effects on both ER α and AhR target genes by direct binding to ERE and XRE sites in these genes and stimulating their up-regulated expression. Our findings that the actions of BEs are mediated through both ER α and AhR, imply that both receptors need to be considered as regulators of the biology and pharmacology of BEs. Of note, as well, the actions of BEs on AhR differed from that of E2. These results provide information useful for understanding potential activities of botanical dietary supplements on target cells in postmenopausal women consuming these dietary supplements. Based on our observations in breast cancer cells and in hepatocytes and hepatoma cells, consuming BEs could up-regulate key target genes of AhR that encode certain cytochrome P450 enzymes. Given that these drug metabolizing enzymes can affect the pharmacokinetics and consequently the potency of many pharmaceuticals, consumption of BEs might result in drug interactions that could alter the effectiveness of important pharmaceuticals, aspects that should be further examined in future work.

Acknowledgments

This research was supported by NIH grant P50AT006268 from the National Center for Complementary and Integrative Health (NCCIH), the Office of Dietary Supplements (ODS) and the National Cancer Institute (NCI) (to BSK), and NIH grant DK015556 (to JAK), and a grant from the Breast Cancer Research Foundation (BCRF) (to BSK). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCCIH, ODS, NCI, or the National Institutes of Health.

Abbreviations

AhR	aryl hydrocarbon receptor
BE	botanical estrogen
CH	the AhR antagonist CH223191
ERα	estrogen receptor- α
ERE	estrogen response element
ICI	the antiestrogen ICI 182,780
SERM	selective estrogen receptor modulator
siRNA	short interfering RNA

XRE xenobiotic response element

References

- Ahmed S, Al-Saigh S, Matthews J. FOXA1 is essential for aryl hydrocarbon receptor-dependent regulation of cyclin G2. *Mol. Cancer Res.* 2012; 10:636–648. [PubMed: 22596188]
- Ahmed S, Valen E, Sandelin A, Matthews J. Dioxin increases the interaction between aryl hydrocarbon receptor and estrogen receptor alpha at human promoters. *Toxicol. Sci.* 2009; 111:254–266. [PubMed: 19574409]
- Beischlag TV, Perdew GH. ER alpha-AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin-inducible gene transcription. *J. Biol. Chem.* 2005; 280:21607–21611. [PubMed: 15837795]
- Bialesova L, Novotna A, Macejova D, Brtko J, Dvorak Z. Agonistic effect of selected isoflavones on arylhydrocarbon receptor in a novel AZ-AhR transgenic gene reporter human cell line. *Gen. Physiol. Biophys.* 2015; 34:331–334. [PubMed: 25926549]
- Boonmuen N, Gong P, Ali Z, Chittiboyina AG, Khan I, Doerge DR, Helferich WG, Carlson KE, Martin T, Piyachaturawat P, Katzenellenbogen JA, Katzenellenbogen BS. Licorice root components in dietary supplements are selective estrogen receptor modulators with a spectrum of estrogenic and anti-estrogenic activities. *Steroids.* 2016; 105:42–49. [PubMed: 26631549]
- Cassidy A, Albertazzi P, Lise Nielsen I, Hall W, Williamson G, Tetens I, Atkins S, Cross H, Manios Y, Wolk A, Steiner C, Branca F. Critical review of health effects of soybean phyto-oestrogens in post-menopausal women. *Proc. Nutr. Soc.* 2006a; 65:76–92. [PubMed: 16441947]
- Cassidy A, Brown JE, Hawdon A, Faughnan MS, King LJ, Millward J, Zimmer-Nechemias L, Wolfe B, Setchell KD. Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. *J. Nutr.* 2006b; 136:45–51. [PubMed: 16365057]
- Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* 2003; 43:309–334. [PubMed: 12540743]
- Denison MS, Soshilov AA, He G, DeGroot DE, Zhao B. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol. Sci.* 2011; 124:1–22. [PubMed: 21908767]
- Doerge DR, Chang HC, Churchwell MI, Holder CL. Analysis of soy isoflavone conjugation *in vitro* and in human blood using liquid chromatographymass spectrometry. *Drug Metab. Dispos.* 2000; 28:298–307. [PubMed: 10681374]
- DuSell CD, Nelson ER, Wittmann BM, Fretz JA, Kazmin D, Thomas RS, Pike JW, McDonnell DP. Regulation of aryl hydrocarbon receptor function by selective estrogen receptor modulators. *Mol. Endocrinol.* 2010; 24:33–46. [PubMed: 19901195]
- Endler A, Chen L, Zhang J, Xu GT, Shibasaki F. Binding of the ERalpha and ARNT1 AF2 domains to exon 21 of the SRC1 isoform SRC1e is essential for estrogen- and dioxin-related transcription. *J. Cell Sci.* 2012; 125:2004–2016. [PubMed: 22328528]
- Ferrante MC, Amero P, Santoro A, Monnolo A, Simeoli R, Di Guida F, Mattace Raso G, Meli R. Polychlorinated biphenyls (PCB 101, PCB 153 and PCB 180) alter leptin signaling and lipid metabolism in differentiated 3T3-L1 adipocytes. *Toxicol. Appl. Pharmacol.* 2014; 279:401–408. [PubMed: 24978599]
- Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS. Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res.* 2004; 64:1522–1533. [PubMed: 14973112]
- Gong P, Madak-Erdogan Z, Li J, Cheng J, Greenlief CM, Helferich W, Katzenellenbogen JA, Katzenellenbogen BS. Transcriptomic analysis identifies gene networks regulated by estrogen receptor alpha (ERalpha) and ERbeta that control distinct effects of different botanical estrogens. *Nucl. Recept. Signal.* 2014; 12:e001. [PubMed: 25363786]

- Hernandez-Ochoa I, Gao L, Peretz J, Basavarajappa MS, Bunting SL, Karman BN, Paulose T, Flaws JA. Follicle-stimulating hormone responsiveness in antral follicles from aryl hydrocarbon receptor knockout mice. *Reprod. Biol. Endocrinol.* 2013; 11:26. [PubMed: 23548098]
- Jiang Y, Gong P, Madak-Erdogan Z, Martin T, Jeyakumar M, Carlson K, Khan I, Smillie TJ, Chittiboyina AG, Rotte SC, Helferich WG, Katzenellenbogen JA, Katzenellenbogen BS. Mechanisms enforcing the estrogen receptor beta selectivity of botanical estrogens. *FASEB J.* 2013; 27:4406–4418. [PubMed: 23882126]
- Kanamori H, Krieg S, Mao C, Di Pippo VA, Wang S, Zajchowski DA, Shapiro DJ. Proteinase inhibitor 9, an inhibitor of granzyme B-mediated apoptosis, is a primary estrogen-inducible gene in human liver cells. *J. Biol. Chem.* 2000; 275:5867–5873. [PubMed: 10681578]
- Krieg AJ, Krieg SA, Ahn BS, Shapiro DJ. Interplay between estrogen response element sequence and ligands controls *in vivo* binding of estrogen receptor to regulated genes. *J. Biol. Chem.* 2004; 279:5025–5034. [PubMed: 14617632]
- Linden J, Lensu S, Pohjanvirta R. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on hormones of energy balance in a TCDD-sensitive and a TCDD-resistant rat strain. *Int. J. Mol. Sci.* 2014; 15:13938–13966. [PubMed: 25119860]
- Liu S, Abdelrahim M, Khan S, Ariazi E, Jordan VC, Safe S. Aryl hydrocarbon receptor agonists directly activate estrogen receptor alpha in MCF-7 breast cancer cells. *Biol. Chem.* 2006; 387:1209–1213. [PubMed: 16972788]
- Lonard DM, O'Malley BW. Nuclear receptor coregulators: modulators of pathology and therapeutic targets. *Nat. Rev. Endocrinol.* 2012; 8:598–604. [PubMed: 22733267]
- Madak-Erdogan Z, Gong P, Katzenellenbogen BS. Differential utilization of nuclear and extranuclear receptor signaling pathways in the actions of estrogens, SERMs, and a tissue-selective estrogen complex (TSEC). *J. Steroid Biochem. Mol. Biol.* 2016a; 158:198–206. [PubMed: 26689478]
- Madak-Erdogan Z, Gong P, Zhao YC, Xu L, Wrobel KU, Hartman JA, Wang M, Cam A, Iwaniec UT, Turner RT, Twaddle NC, Doerge DR, Khan IA, Katzenellenbogen JA, Katzenellenbogen BS, Helferich WG. Dietary licorice root supplementation reduces diet-induced weight gain, lipid deposition, and hepatic steatosis in ovariectomized mice without stimulating reproductive tissues and mammary gland. *Mol. Nutr. Food Res.* 2016b; 60:369–380. [PubMed: 26555669]
- Madak-Erdogan Z, Katzenellenbogen BS. Aryl hydrocarbon receptor modulation of estrogen receptor alpha-mediated gene regulation by a multimeric chromatin complex involving the two receptors and the coregulator RIP140. *Toxicol. Sci.* 2012; 125:401–411. [PubMed: 22071320]
- Maximov PY, Lee TM, Jordan VC. The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice. *Curr. Clin. Pharmacol.* 2013; 8:135–155. [PubMed: 23062036]
- McDonnell DP, Wardell SE. The molecular mechanisms underlying the pharmacological actions of ER modulators: implications for new drug discovery in breast cancer. *Curr. Opin. Pharmacol.* 2010; 10:620–628. [PubMed: 20926342]
- McKenna NJ, Evans RM, O'Malley BW. Nuclear receptor signalling: a home for nuclear receptor and coregulator signaling research. *Nucl. Recept. Signal.* 2014; 12:e006. [PubMed: 25614732]
- Morton MS, Wilcox G, Wahlqvist ML, Griffiths K. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *J. Endocrinol.* 1994; 142:251–259. [PubMed: 7930998]
- O'Donnell EF, Koch DC, Bisson WH, Jang HS, Kolluri SK. The aryl hydrocarbon receptor mediates raloxifene-induced apoptosis in estrogen receptor-negative hepatoma and breast cancer cells. *Cell Death Dis.* 2014; 5:e1038. [PubMed: 24481452]
- Saito R, Miki Y, Hata S, Takagi K, Iida S, Oba Y, Ono K, Ishida T, Suzuki T, Ohuchi N, Sasano H. Aryl hydrocarbon receptor in breast cancer-a newly defined prognostic marker. *Horm. Cancer.* 2014; 5:11–21. [PubMed: 24078229]
- Santen RJ, Allred DC, Ardoin SP, Archer DF, Boyd N, Braunstein GD, Burger HG, Colditz GA, Davis SR, Gambacciani M, Gower BA, Henderson VW, Jarjour WN, Karas RH, Kleerekoper M, Lobo RA, Manson JE, Marsden J, Martin KA, Martin L, Pinkerton JV, Rubinow DR, Teede H, Thiboutot DM, Utian WH. Postmenopausal hormone therapy: an Endocrine Society scientific statement. *J. Clin. Endocrinol. Metab.* 2010; 95:s1–s66. [PubMed: 20566620]

- Schmidt JV, Su GH, Reddy JK, Simon MC, Bradfield CA. Characterization of a murine AhR null allele: involvement of the Ah receptor in hepatic growth and development. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:6731–6736. [PubMed: 8692887]
- Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, Brashear WT, Kirschner AS, Cassidy A, Heubi JE. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* 2001; 131:1362s–1375s. [PubMed: 11285356]
- Simpson E, Santen RJ. Celebrating 75 years of oestradiol. *J. Mol. Endocrinol.* 2015; 55:T1–T20. [PubMed: 26438567]
- Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr. Rev.* 2004; 25:45–71. [PubMed: 14769827]
- Spencer JP, Abd-el-Mohsen MM, Rice-Evans C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch. Biochem. Biophys.* 2004; 423:148–161. [PubMed: 14989269]
- Spink BC, Bennett JA, Lostritto N, Cole JR, Spink DC. Expression of the aryl hydrocarbon receptor is not required for the proliferation, migration, invasion, or estrogen-dependent tumorigenesis of MCF-7 breast cancer cells. *Mol. Carcinog.* 2013; 52:544–554. [PubMed: 22388733]
- Stender JD, Kim K, Charn TH, Komm B, Chang KC, Kraus WL, Benner C, Glass CK, Katzenellenbogen BS. Genome-wide analysis of estrogen receptor alpha DNA binding and tethering mechanisms identifies Runx1 as a novel tethering factor in receptor-mediated transcriptional activation. *Mol. Cell. Biol.* 2010; 30:3943–3955. [PubMed: 20547749]
- Timsit YE, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. *Steroids.* 2007; 72:231–246. [PubMed: 17284330]
- Tiong CT, Chen C, Zhang SJ, Li J, Soshilov A, Denison MS, Lee LS, Tam VH, Wong SP, Xu HE, Yong EL. A novel prenylflavone restricts breast cancer cell growth through AhR-mediated destabilization of ERalpha protein. *Carcinogenesis.* 2012; 33:1089–1097. [PubMed: 22345291]
- Van der Heiden E, Bechoux N, Muller M, Sergeant T, Schneider YJ, Larondelle Y, Maghuin-Rogister G, Scippo ML. Food flavonoid aryl hydrocarbon receptor-mediated agonistic/antagonistic/synergic activities in human and rat reporter gene assays. *Anal. Chim. Acta.* 2009; 637:337–345. [PubMed: 19286049]
- Wahlang B, Falkner KC, Gregory B, Ansert D, Young D, Conklin DJ, Bhatnagar A, McClain CJ, Cave M. Polychlorinated biphenyl 153 is a diet-dependent obesogen that worsens nonalcoholic fatty liver disease in male C57BL6/J mice. *J. Nutr. Biochem.* 2013; 24:1587–1595. [PubMed: 23618531]
- Wardell SE, Kazmin D, McDonnell DP. Research resource: transcriptional profiling in a cellular model of breast cancer reveals functional and mechanistic differences between clinically relevant SERM and between SERM/estrogen complexes. *Mol. Endocrinol.* 2012; 26:1235–1248. [PubMed: 22570330]
- Xu X, Harris KS, Wang HJ, Murphy PA, Hendrich S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* 1995; 125:2307–2315. [PubMed: 7666247]

Fig. 1. Comparison of the ability of E2, TCDD, and botanical estrogens (BEs) to up-regulate and/or down-regulate ER α and AhR target genes

Progesterone receptor (PgR), pS2, and CYP1A1 and CYP1B1 are differently regulated by these compounds. MCF-7 cells were treated with 10^{-8} M E2 or 10^{-9} M TCDD or 10^{-6} M BE for 4 h and mRNA levels of the ER α target genes, PgR and pS2 (panel A) and the AhR target genes CYP1A1 and CYP1B1 (panel B) were monitored by qPCR mRNA levels were normalized relative to the housekeeping gene 36B4, and fold change calculated relative to the control vehicle treated samples. Results are the average $\pm$ SD from at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$.

Fig. 2. Dose-response relationships for regulation of the ER α target gene, PgR, and the AhR target gene CYP1A1 by E2, TCDD and four botanical estrogens

MCF-7 cells were treated with the indicated concentrations of compounds for 24 h and gene expression was monitored by qPCR. Note that the CYP1A1 mRNA scale (at right) differs in the panels to highlight the different up- and down-regulations by the different compounds. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 3. Use of ER α and AhR inhibitors and the siRNA knockdown of ER α or AhR to analyze receptor mediation of the effects of BEs, E2, and TCDD on gene regulation

A. MCF-7 cells were treated with the indicated compounds or control vehicle for 4 h. For treatment with inhibitors, cells were pretreated with 10^{-6} MICI 182,780 or 10^{-6} M CH 223191 for 45min before cotreatment with 10^{-8} M E2 or 10^{-6} M BE + ICI or + CH for an additional 4 h prior to RNA harvest and gene expression determination by qPCR. **B.** MCF-7 cells were transfected with 25 nM of control or ER α siRNA or AhR siRNA for 48 h and were then treated with the indicated compounds for 4 h prior to RNA harvest. Western blot inset in lowest right panel shows selective knockdown of ER α or AhR by treatment with their respective siRNAs. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 4. Assessment of the activation of AhR and ER α target genes, by BEs, E2, and TCDD in primary hepatocytes from wild type mice or AhR-knockout mice and in HepG2 cells stably expressing ER α .

A. Mouse primary hepatocytes were prepared and pooled from livers of at least two littermate mice with the same genotype. Cells were cultured for 24 h and then treated with vehicle, 10^{-8} M EE2, or 10^{-9} M TCDD or BE for an additional 24 h prior for determination of gene expression by qPCR. **B.** HepG2 cells stably expressing ER α (HepG2-ER α) were cultured with 0.1% ethanol vehicle; 10^{-9} M EE2; 10^{-9} M TCDD; or 10^{-6} M Genistein, Daidzein, S-equol or liquiritigenin alone or in the copresence of 10^{-6} M antiestrogen ICI 182,780 or AhR antagonist CH 223,191 for 24 h prior to determination of expression of the ER α target gene PI9, and the AhR target gene, CYP1A1 by qPCR. Values are mean $\pm$ SD of 3 determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 5. Impact of E2, TCDD, and botanical estrogens on MCF-7 cell proliferation

A. Ki67 mRNA level monitored after 24 h of control vehicle or compound (10^{-8} M E2, 10^{-9} M TCDD or 10^{-6} M BE) treatment in the absence or presence of 10^{-6} M ICI 182,780 or 10^{-6} M CH 223191. **B.** Cell proliferation monitored after 6 days of treatment with the indicated concentrations of E2 or TCDD. **C.** Cell proliferation monitored at 6 days after treatment with veh, 10^{-8} M E2, 10^{-9} M TCDD or 10^{-6} M BE alone or with 10^{-6} M ICI 182,780 or 10^{-6} M CH 223191. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 6. Recruitment of ERα vs. AhR to (A) estrogen responsive (estrogen response element-containing) regions of the TFF1/pS2 and LRRC54 estrogen target genes, and (B) to xenobiotic response element-containing regions of the CYP1A1 or CYP1B1 genes
MCF-7 cells were treated with 10^{-8} M E2, 10^{-9} M TCDD, or 10^{-6} M liquiritigenin or 10^{-6} M S-equol for 45 min prior to chromatin crosslinking and chromatin immunoprecipitation (ChIP) using control IgG antibody, ERα antibody, or AhR antibody. Receptor recruitment is presented as percent of input. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 7. The effect of co-treatment with E2 or BEs plus TCDD on the expression of ERα target genes, PgR and pS2, and AhR target genes, CYP1A1 and CYP1B1
MCF-7 cells were treated with Veh, 10^{-8} M E2 or 10^{-6} M BE alone (open bars) or with 10^{-9} M TCDD (grey bars) for 24 h and mRNA levels of the ERα target genes, PgR and pS2 (Panel A) and the AhR target genes CYP1A1 and CYP1B1 (Panel B) were monitored by qPCR. Student's *t*-test was performed on the data in Panel A to compare E2 or BE treatment alone vs. treatment plus TCDD. One-way ANOVA was performed on the data in Panel B to compare cotreatment with E2 or BE plus TCDD vs. TCDD treatment alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant.

Fig. 8. Schematic model depicting the specificity and crosstalk between ER α and AhR in mediating the differential actions of the ER α ligand E2, and the AhR ligand TCDD, and the dual acting botanical estrogens (BEs)

E2 and TCDD largely activate only their own receptors, ER α and AhR, respectively, with crosstalk by E2-ER α suppressing the activity of TCDD-AhR. By contrast, BEs have dual specificity, activating estrogen target genes via ER α and also dioxin/xenobiotic target genes through AhR, with their AhR activity being modulated by negative crosstalk via ERs. Thus, treatment of cells with ER α antagonist ICI 182,780 or depletion of cellular ER α by siRNA-mediated knockdown enhanced the magnitude of stimulation of AhR target genes by BEs. The suppressive effect of the estrogens acting through ER α on the expression of AhR target

genes could be due to either a direct competitive binding effect between ER α and AhR at the chromatin level or could be due to more indirect effects via ER α .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

PCR primers used in this study.

			Sequence	
			Forward	Reverse
Real-time PCR primers	PgR	Human	ACCCGCCCTATCTCAACTACC	AGGACACCATAATGACAGCCT
	TFF1/pS2	Human	CCCGTGAAAGACAGAATTGT	GGTGTCGTCGAAACAGCAG
	PI9	Human	AATGCAAGTGGTACTTTTGCCA	AAGCCCGATGAATGTCTTCCT
	CYP1A1	Human	TCGGCCACGGAGTTTCTTC	GGTCAGCATGTGCCCAATCA
		Mouse	CAATGAGTTTGGGGAGGTTACTG	CCCTTCTCAAATGCCTGTAGTG
	CYP1B1	Human	TGAGTGCCGTGTGTTTCGG	GTTGCTGAAGTTGCGGTTGAG
		Mouse	CCACCAGCCTTAGTGCAGAC	GGCCAGGACGGAGAAGAGT
	Leptin	Mouse	GTGGCTTTGGTCCTAICTGTC	CGTGTGTGAAATGTCATTGATCC
ChIP primers	pS2_ERE	Human	GCCACCATGGAGAACAAGGT	ACTGGGAGGGCGTGACAC
	LRRC54_ERE	Human	ACGCAACAACCCAAGCATG	GGGAAGATGTCACCGTGACC
	CYP1A1_XRE	Human	CCACCCTTCGACAGTTCCT	GCGTTGCGTGAGAAGGAC
	CYP1B1_XRE	Human	ATATGACTGGAGCCGACTTCC	GGCGAACTTTATCGGGTTGA

Table 2

EC₅₀ values for regulation of progesterone receptor (PgR) and CYP1A1 gene expression by E2, TCDD, and four botanical estrogens.

Compound	EC ₅₀ (M)	
	PgR	CYP1A1
E2	1.20×10^{-11}	3.0×10^{-12} (IC ₅₀)
TCDD	-	3.73×10^{-10}
Genistein	1.12×10^{-8}	2.17×10^{-6}
Daidzein	1.54×10^{-7}	1.95×10^{-6}
S-Equol	8.89×10^{-8}	3.54×10^{-6}
Liquiritigenin	2.14×10^{-7}	2.18×10^{-6}