Differential Effects of Xenoestrogens on Coactivator Recruitment by Estrogen Receptor (ER) α and ERβ*

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It has been proposed that tissue-specific estrogenic and/or antiestrogenic actions of certain xenoestrogens may be associated with alterations in the tertiary structure of estrogen receptor (ER) α and/or ERβ following ligand binding; changes which are sensed by cellular factors (coactivators) required for normal gene expression. However, it is still unclear whether xenoestrogens affect the normal behavior of ERα and/or ERβ subsequent to receptor binding. In view of the wide range of structural forms now recognized to mimic the actions of the natural estrogens, we have assessed the ability of ERα and ERβ to recruit TIF2 and SRC-1a in the presence of 17β-estradiol, genistein, diethylstilbestrol, 4-tert-octylphenol, 2',3',4',5'-tetrachlorobiphenyl-ol, and bisphenol A. We show that ligand-dependent differences exist in the ability of ERα and ERβ to bind coactivator proteins in vitro, despite the similarity in binding affinity of the various ligands for both ER subtypes. The enhanced ability of ERβ (over ERα) to recruit coactivators in the presence of xenoestrogens was consistent with a greater ability of ERβ to potentiate reporter gene activity in transiently transfected HeLa cells expressing SRC-1a and TIF2. We conclude that ligand-dependent differences in the ability of ERα and ERβ to recruit coactivator proteins may contribute to the complex tissue-dependent agonistic/antagonistic responses observed with certain xenoestrogens.

One of the greatest challenges in understanding the mechanisms of estrogen action has been to determine how different estrogen receptor (ER) ligands (steroidal estrogens, antiestrogens, xenoestrogens) produce such diverse biological effects. The recent discovery of a second subtype of the estrogen receptor (ERb), adds another level of complexity to the mechanism of estrogen action and has opened the possibility of new pathways that might exist expressing both ER isoforms (9, 10), although this remains to be proven in vivo.

Estrogen receptors activate transcription of target genes via two activation functions; AF-1 (in the N-terminal domain) is ligand-independent and is regulated by phosphorylation in response to growth factors (11), whereas AF-2 is closely associated with the LBD and depends on ligand binding for its transcriptional activity. The activities of AF-1 and AF-2 of the ER vary depending upon the responsive promoter (12) and cell type, and in some cases both are required for full transcriptional activation of target genes (13, 14). Although the amino acid homology within the LBD of rat ERα and ERβ does not exceed 55%, a number of residues required for ligand binding and for the formation of the hydrophobic pocket are highly conserved between the two receptor isoforms. Therefore, the reported similarity in the relative binding affinities of ERα and ERβ for a range of natural and synthetic estrogens may have been anticipated (15, 16). However, ERα and ERβ contain a region of relatively low amino acid homology within the LBD, a region that has been shown to be accessible to proteolytic attack (17), and is therefore likely to contain residues on an exposed surface of the receptor, which may be involved in subsequent receptor-protein interactions. The ligand-dependent transactivation domain AF-2 is of particular interest because it has provided a mechanistic explanation for the observed functional differences between agonists and antagonists of the ER. It is now believed that the primary role of 17β-estradiol binding to the ER is to induce a conformational change in the tertiary structure of the ER, which subsequently affects the alignment of a highly conserved amphipathic α-helix (helix 12) within AF-2 (18). Correct alignment of helix 12 (in the presence of 17β-estradiol), exposes residues that interact with other proteins (known as transcriptional intermediary
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Factors or coactivators) necessary for the formation of a stable pre-initiation complex (19), whereas it is misaligned with the estrogen antagonist raloxifene (20). Together, these findings suggest that ERα and/or ERβ may display different profiles for coactivator protein recruitment depending on the structure of the ligand.

A surprising number of proteins have now been identified that exhibit all the properties expected for mediators of AF-2, i.e. (i) they interact in vivo with nuclear receptors in an agonist-dependent manner, (ii) they bind directly to the ligand-binding domain in an agonist- and AF-2-integrity-dependent manner in vitro, (iii) they harbor an autonomous transcriptional activation function, (iv) they relieve nuclear squelching, and (v) they enhance the activity of some nuclear receptor AF-2s when overexpressed in mammalian cells (21). These include RIP140 (22), TRIP1-1/SUG-1 (23, 24), TIF1 (25), SRC-1 (26, 27), TIF2/GRIP-1 (21, 28), ACTR/SRC-3 (29, 30), and CBP/p300 (31). Although the function of some of these remains to be established, the p160 proteins (such as SRC-1 and TIF2) have been shown to potentiate the transcriptional activity of several nuclear receptors in transiently transfected cells (21, 26, 28, 29, 32–34). The recruitment of these proteins to activated receptors is mediated by α-helical LXXLL motifs (34) in which L denotes leucine and X denotes any amino acid.

It was recently shown (using a range of short peptide sequences directed toward the surface of the ER) that a range of natural and synthetic estrogens/anti-estrogens induced distinct conformational changes in the tertiary structure of ERα and/or ERβ (35). Thus, it has been proposed that the tissue-specific estrogenic and/or antiestrogenic actions of certain xenoestrogens may be associated with distinct changes in the tertiary structure of ERα and/or ERβ following ligand binding; changes that are sensed by cellular factors required for normal gene expression. Indeed, ERα was recently reported to display ligand-dependent selectivity for coactivator recruitment in a two-hybrid system (expressing chimeric ERα and coactivator fusion proteins) in yeast (36).

In view of the wide range of chemical structures now known to mimic the actions of the natural estrogens (37), GST pull-down assays were used to assess the ability of both ERα and ERβ to recruit transcriptional intermediary factor-2 (TIF2) and steroid receptor coactivator-1α (SRC-1α) in vitro, with 17β-estradiol, genistein (Gen, a phytoestrogen), diethylstilbestrol (DES, a stilbene), 4-tet-octylphenol (OP, an alkylphenol), 2,3,4,5-tetrachlorobiphenyl-4-ol (PCB-4OH, a PCB metabolite), and bisphenol A (Bis-A, a biphenolic compound). We show that ERα and ERβ differ in terms of their ability to recruit SRC-1α and TIF2 with the various xenoestrogens in vitro, despite the two receptors having relatively similar binding affinities for these compounds. The enhanced ability of ERβ to recruit coactivators in vitro, in the presence of xenoestrogens, was also consistent with the greater capacity of ERβ to potentiate reporter gene expression in transiently transfected HeLa cells carrying expression plasmids for SRC-1α and TIF2 relative to ERα.

MATERIALS AND METHODS

**Chemicals—**17β-Estradiol (>98% pure), genistein (>98% pure), diethylstilbestrol (>99% pure), and bisphenol A (98% pure) were purchased from Sigma (Dorset, United Kingdom (UK)) and were research grade chemicals. 2,3,4,5-Tetrachlorobiphenyl-4-ol (95% pure) was supplied by Greylound Chemical Service (Merseyside, UK). 4-tet-Octylphenol (98% pure) was supplied by Schenectady International Inc. (Schenectady, NY). All stock solutions were made up in MeSO (Sigma).

Expression and Reporter Plasmids—The expression plasmids GST-AF2α, GST-SRC1α, pSG5-SRC1c, pGal4-AF2α (27), GST-AF2β, pGal4-AF2β (38), and pSG5-ERβ (9) have been described previously. pSG5-ERα and pSG5-TIF2 were a gift from Dr. Pierre Chambon. The reporter plasmid pGal4,TK, GL3 (27) is based on the pGL3 (firefly luciferase) series of vectors (Promega), and the internal control was the Renilla luciferase plasmid pRLCMV. The amount of DNA used in the transfection assays was adjusted using pMT2 (22).

**Coupled In Vitro Transcription and Translation—**All in vitro translations were performed using a TNT coupled rabbit reticulocyte lysate system, with the appropriate RNA polymerase (T7), according to the manufacturer's instructions (Promega). Radiolabeled proteins were synthesized by substituting 1 mM methionine with [35S]methionine (Amersham Pharmacia Biotech) in the reaction mixture. Radiolabeled products from the in vitro translations were analyzed by 8% (25-TIF2) or 10% (35S-ERα and 35S-ERβ) SDS-PAGE, respectively.

**GST Pull-down Assays—**Two types of GST pull-down assays were used to assess the ability of ERα and ERβ to recruit SRC-1α or TIF2 following binding to a range of xenoestrogens. The first employed GST-AF2α or GST-AF2β fusion proteins with [35S]methionine-radiolabeled translation products (translated in parallel) were separated on 10% SDS-polyacrylamide gels in order to check the integrity of the ER translation products. Translation products were diluted 20-fold in ligand binding buffer (20 mM Hepes, pH 7.4, 1.5 mM EDTA, 0.1% bovine serum albumin, 0.25 mM dithiothreitol, 10% glycerol) and kept on ice. Aliquots (45 μl) of 20-fold diluted receptor preparation were pre-washed with 50 μl of [2,4,6,7-3H](estradiol (evaporated to dryness in the tube) and incubated in the presence of various concentrations of competitor (5 μl of chemical in MeSO) for at least 16 h at 4 °C. The final incubation volume was 50 μl. Free and bound radioligand were separated by adding 50 μl of ice-cold DCC (0.1 g of dextran T70, 1.0 g of activated charcoal, 4.0 ml of 1 × Tris, pH 7.4, 0.8 ml of 0.5 mM EDTA, made up to a final volume of 400 ml in DW) to each reaction tube. The tubes were mixed briefly, incubated on ice for 5 min, and centrifuged for 5 min at 4 °C to pellet the charcoal. The supernatant was removed and added directly to β-lys (Pony Vial™, Packard) containing scintillant (Liquisint™, National Diagnostics). Bound radioactivity was measured using a TRI-CARB® 2000CA liquid scintillation analyzer (Packard®). Specific binding in the presence of competitor was expressed as a percentage of the maximum binding (calculated by subtracting the nonspecific binding from the total binding). Receptor binding affinity (BBA) was calculated as the ratio of concentrations of E2 or competitor required to reduce the specific radioligand binding by 50% (BBA value for E2 was arbitrarily set at 100 for both receptors).

**GST Pull-down Assays—**Two types of GST pull-down assays were used to assess the ability of ERα and ERβ to recruit SRC-1α or TIF2 following binding to a range of xenoestrogens. The first employed GST-AF2α or GST-AF2β fusion proteins with [35S]methionine-radiolabeled translation products translated 35S-TIF2, and the second employed a GST-SRC1α fusion protein with [35S]methionine-radiolabeled translation products translated in vitro 35S-ERα or 35S-ERβ (see Fig. 1).

The expression and purification of GST fusion proteins was as described previously (39). In brief, overnight cultures of E. coli (BL21 DE3) containing the GST expression plasmid were incubated 1:10 in L-Broth medium containing ampicillin (final concentration, 100 mg/liter). The cultures were incubated for an additional 1 h at 37 °C, after which the cultures were induced by adding isopropyl β-D-thiogalactosidase (0.1 mM final concentration). After 4 h, bacteria were collected by centrifugation, resuspended, and concentrated 10-fold in NETN (0.5% Nonidet P-40, 10 mM EDTA, 20 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol) containing protease inhibitors (5 μg of leupeptin/ml, 5 μg of pepstatin/ml, 40 μg of phenylmethylsulfonyl fluoride/ml, 1 mM dithiothreitol, 2 μg of aprotinin/ml), sonicated, and centrifuged (~5000 × g, 20 min, 4 °C). Bacterial lysates containing the GST fusion proteins were stored in capped Falcon tubes (10-ml aliquots) at −70 °C until required.

Glutathione-Sepharose beads (Amersham Pharmacia Biotech) were pre-washed in NETN containing protease inhibitors (NETN + P) and 0.5% powdered milk (to minimize nonspecific binding), and were resuspended in one volume of NETN + P. Fusion proteins (GST-AF2α or GST-SRC1α) contained within the bacterial lysates were purified onto the pre-washed glutathione-Sepharose beads (25 μl of beads/ml of lysate) by incubation for 1.5 h at 4 °C on a rotary mixer. The beads (loaded with GST-fusion proteins) were collected by centrifugation, washed four times with NETN + P, and resuspended in one volume of NETN + P prior to use. 50 μl of beads, containing GST fusion proteins or GST alone (negative control), were incubated overnight at 4 °C in Eppendorf tubes containing 845 μl of NETN + P, 80 μl of MeSO, and 15 μl of the in vitro translated [35S]-labeled receptor or coactivator (Fig. 1), in the presence of 10 μl of vehicle or test chemical (in MeSO). The beads were then
washed four times with NETN (all supernatant removed in final wash),
dried in a Speed Vac for 30 min, resuspended in 61 µl of 2× protein
loading buffer (reducing), and boiled for 4 min to release all the bound
liquid scintillation analyzer (Packard®). SDS-PAGE gels were fixed and
values from a representative experiment are presented.

The RRA value for E2 was arbitrarily set at 100 for both receptors.

Cell Transfection Assay—HeLa cells were plated in 96-well microti-
ner plates in phenol red-free medium, and were incubated overnight at
37 °C to reach approximately 30% confluence. Cells were transfected
using the calcium phosphate coprecipitation method, as described pre-
viously (18). The transfected DNA included a reporter plasmid (pGal4-Luc; Packard®) containing scintillant (Liquiscint®; National Diag-
nostics), and the radioactivity was counted using a TRI-CARB® 2000CA
liquid scintillation analyzer (Packard®). SDS-PAGE gels were fixed and
dried in a Speed Vac for 30 min, resuspended in 61 µl of Renlite reagent (1 mg/ml coelenterazine stock

RESULTS

Specificity of ERα and ERβ to a Range of Xenoestrogens—
The binding affinities of E2, DES, Gen, PCB-OH, OP, and Bis-A for ERα and ERβ were assessed by their ability to compete with [2,4,6,7-3H]17β-estradiol for binding to in vitro translated receptors over a 100,000-fold concentration range (Fig. 2). All of the chemicals tested were able to compete with tritiated 17β-estradiol for binding to both ERα and ERβ in a dose-dependent manner. RBA for ERα and ERβ were 53 and 150 for DES, 0.7 and 15 for Gen, 1.6 and 3.0 for PCB-OH, 0.013 and 0.25 for OP,
and 0.075 and 0.75 for Bis-A, respectively (Table I). ERα always had a greater RBA for all the xenoestrogens tested compared with ERβ. The largest differences in RBA were seen with Gen and OP, which were approximately 20-fold higher with ERβ than ERα (Table 1).

ERα and ERβ Differ in Their Ability to Recruit Coactivator Proteins following Xenoestrogen Binding—The ability of either ERα or ERβ fusion proteins to bind TIF2 and SRC-1a with different concentrations of ligand was assessed using two GST pull-down assay systems. Thus, the ability of GST-LBD to bind [35S]methionine-labeled TIF2 or SRC-1a was examined, and conversely the ability of GST-SRC-1 to bind [35S]methionine-labeled ERα or ERβ was tested. Fig. 3 shows a typical autorad from a GST pull-down assay, where the dose-dependent recruitment of [35S]-TIF2 by GST-AF2α with E2 and DES is demonstrated. All of the chemicals tested enabled ERα and ERβ to bind TIF2 and SRC-1a in a dose-dependent manner with one exception; ERα was not able to bind either TIF2 or SRC-1a in the presence of PCB-OH over the concentration

FIG. 1. Two GST pull-down assays were used to investigate the
receptor-coactivator interactions in the presence of different
ligands. The ability of radiolabeled TIF2 to interact with the hER
was investigated using a GST fusion protein containing the LBD and AF-2 of either hERα or hERβ (A). In contrast, the ability of radiolabeled ERα or ERβ (entire protein) to interact with SRC-1a was investigated using a GST-SRC-1a fusion protein (B). The GST fusion proteins associate with the beads, and the ligands interact with the LBD of the ER. Ligand-induced conformational changes in the tertiary structure of the estrogen receptor are associated with differences in the receptor’s ability to interact with coactivator proteins (TIF2 or SRC-1a).

FIG. 2. Ligand binding assays. Competitive displacement of tritiated 17β-estradiol from in vitro translated hERα (A) and hERβ (B) by a range of xenoestrogens. Unbound radioligand was removed after incubation (16 h at 4 °C) as described. Specific binding was calculated by subtracting the nonspecific counts from the total counts, and this was expressed as a percentage of the maximum obtainable response (Max. Binding) with excess tritiated 17β-estradiol alone (value arbitrarily set at 100%). Values represent the mean ± S.E. of four separate experiments.

The ability of radiolabeled TIF2 to interact with the hER was
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range tested (Figs. 4 and 5, respectively). In contrast, ERβ was able to bind TIF2 (albeit submaximally; <25% of the maximal inducible response) and SRC-1a with PCB-OH (Figs. 4B and 5B). The RRA of ERα and ERβ for TIF2 were 11 and 60 for DES, 0.005 and 60 for Gen, 0.0002 and 0.2 for OP, and <0.0001 and 0.05 for Bis-A, respectively (Table II). The RRA of ERα and ERβ for SRC-1a were 5 and 50 for DES, 0.06 and 2 for Gen, 0 and 0.05 for PCB-OH, 0.002 and 0.002 for OP, and 0.0003 and 0.0002 for Bis-A, respectively (Table II). The 20-fold greater affinity of ERβ for Gen (Table I; Fig. 2) resulted in a 12,000- and 33-fold greater ability to bind TIF2 and SRC-1a, respectively, relative to ERα (Table II; Figs. 4 and 5). In contrast, the abilities of ERα and ERβ to bind SRC-1a with octylphenol and bisphenol A were similar (Table II), despite the greater binding affinities of these compounds for ERβ (Table I).

**Mammalian Cell Transfection Assay**—The consequence of differences in the ability of ERα and ERβ to bind coactivator proteins (as shown in the GST pull-down assays) on gene expression was assessed in transiently transfected HeLa cells, using expression vectors encoding the DNA-binding domain of Gal4 fused to the AF-2 domains of ERα or ERβ. These chimeric receptors recognize and stimulate transcription from the pGal4cTGL3 reporter gene construct in the presence of ligand. As the magnitude of the transcriptional response was found to vary between AF2α and AF2β (i.e. pGal4-AF2α produced a response that was approximately 2–3 times greater than that of pGal4-AF2β), the changes in the transcriptional response were expressed relative to the maximal inducible response observed with E2 (10−8 M) in the absence of excess coactivator (response arbitrarily set at 100%). Differences in the base-line response for ERα/β in the absence of hormone (NH) with excess coactivator required that potentiation (or -fold increases) of reporter gene activity were calculated from their respective base line in each case. The base-line response for Gal4-AF2β in the absence of hormone was not resolved above the background level of the assay. This is evident from the raw data, where the NH response of ERα without coactivator (14.0 ± 4.1 luciferase units) occurred above the background level (4.4 ± 1.8 luciferase units), whereas the NH response of ERβ without coactivator (4.4 ± 2.1 luciferase units) did not (data not shown). Therefore, the calculated percentage of response of ERβ in the absence of hormone and coactivator was overestimated, and the resultant -fold increase in reporter gene activity (calculated using this baseline value) with ERβ was underestimated. Consequently, we were unable to determine the true increase in reporter gene activity with ERβ in this instance, and therefore potentiation of reporter gene activity in the absence of excess coactivator was not presented.

Figs. 6–8 show the potentiation (expressed as a -fold increase above the respective base line) of reporter gene activity by ERα and ERβ in transiently transfected HeLa cells carrying expression plasmids for SRC-1a and TIF2 exposed to xenoestrogens and E2. In all cases, potentiation of reporter gene activity was greater with ERβ than ERα. This is consistent with the results from the pull-down assay, in which the RRA values of ERβ for SRC-1a and TIF2 with each xenoestrogen always exceeded those of ERα (Table II). The magnitude of reporter gene potentiation was also associated with the RRA values calculated in the pull-down assays (Table II). For example, in the presence of ERβ, genistein (10−6 M) was able to potentiate reporter gene activity to levels around 72% and 86% of those produced by E2 (10−8 M) with SRC-1a (RRA of 33) and TIF2 (RRA of 60), respectively. In contrast, potentiation of reporter gene expression by PCB-OH and Bis-A (which had lower RRA values) produced only a 3% and 1% increase in reporter gene activity, respectively.
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**Fig. 5.** Results of GST pull-down assay showing the dose-dependent recruitment of SRC-1a by ERα (A) and ERβ (B) complexed to a range of xenoestrogens. In vitro translated 35S-ERα or 35S-ERβ proteins were incubated with glutathione-Sepharose beads carrying GST-SRC1a fusion proteins with various concentrations of E2, DES, PCB-OH, Gen, OP, or Bis-A. Receptor-coactivator recruitment was quantified by scintillation counting as described. Results are expressed arbitrarily as a percentage of the maximum inducible response in the assay. The responses shown are representative of at least four independent experiments, which gave similar results. Values did not generally exceed 50% of the maximum inducible response for both ERα and ERβ even at higher concentrations.

**DISCUSSION**

The primary purpose of 17β-estradiol binding to the ER is to induce a conformational change in the tertiary structure of the ER, such that AF-2 is in a position to mediate the assembly of the basal transcription machinery following the recruitment of coactivators or transcription initiation factors (18). However, the actual conformational change in the tertiary structure of the ER induced by xenoestrogens may differ from that of 17β-estradiol (35) due to differences in the steric and electrostatic properties of the various ligands. In this study, we show that binding of a range of natural and synthetic xenoestrogens to ERα and ERβ alters their abilities (to different extents) to recruit coactivator proteins in vitro, and this may in turn affect their abilities to potentiate the expression of a reporter gene in transiently transfected HeLa cells. In short, ligand-dependent differences in the ability of ERα and ERβ to recruit coactivator proteins may also contribute to the complex tissue-dependent responses observed with certain xenoestrogens.

Receptor-binding assays and GST pull-down assays were used to compare the affinities of xenoestrogens for both ER subtypes with their subsequent abilities to recruit SRC-1a and TIF2 in vitro. We found that all of the xenoestrogens tested were able to displace tritiated 17β-estradiol from ERα/β in a dose-dependent manner. The binding affinity of genistein for ERβ was 20-fold higher than ERα, which was consistent with previous findings (16). Although it is not possible to compare the recruitment profiles of the two GST pull-down assays directly (because the two systems are not analogous; see Fig. 1), it was interesting to note that the RBA of the various xenoestrogens for ERα/β were not always consistent with their subsequent ability to recruit coactivator proteins. This finding may explain previous reports that there is not always a direct correlation between binding affinity and transcriptional potency with certain ER ligands (40). With most of the xenoestrogens tested, ERα and ERβ were able to recruit TIF2 and SRC-1a in a dose-dependent manner. However, ERα was unable to recruit TIF2 and SRC-1a, and ERβ displayed submaximal recruitment of TIF2, with PCB-OH. In contrast, ERβ was able to recruit SRC-1a fully (and in a dose-dependent manner) with PCB-OH. The observed differences in the ability of ERα/β to recruit TIF2 and SRC-1a with PCB-OH were not anticipated (Table I), given the similar RBA of this compound for both ER subtypes (Table I). The 20-fold selective affinity of genistein for ERβ (Table I) resulted in a 12,000- and 33-fold greater ability of ERβ to recruit SRC-1a and TIF2, respectively, compared with ERα (Table II). In contrast, the ability of ERα and ERβ to recruit SRC-1a with octylphenol and bisphenol A were similar, despite the higher binding affinities of these two compounds for ERβ. In general, ERβ had a greater RRA for SRC-1a and TIF2 than ERα with all the xenoestrogens tested. However, unlike their RBAs (which only varied by up to 1 order of magnitude between ERα and ERβ), their RRAs differed by as much as 4 orders of magnitude (Tables I and II). Thus, receptor binding affinities of xenoestrogens for ERα and ERβ may not accurately predict the receptors’ subsequent abilities to recruit different coactivator proteins. Certain coactivators appear to be differentially expressed among tissues, suggesting that they may be involved in the regulation of tissue-selective gene expression. As an example, the coactivator SRC-3 is abundant in the mammary gland and uterus (30), and has a higher affinity for ERα (relative to ERβ), which is the predominant ER form in these tissues. Moreover, it was recently shown that the ability of ERβ to stimulate ERE-TK-Luc reporter gene expression in transiently transfected cells in the presence of 17β-estradiol was dependent on the cell line used (38). Together, these findings suggest that (promoter context aside) the behavior of the ER subtypes within any given cell or tissue will be influenced by the type and level of the accessory proteins present. Thus, in the context of the whole tissue, the results from the GST pull-down assays may imply that genistein would be most effective in cells containing ERβ, and where TIF2 is the predominant coactivator present. In contrast, PCB-OH may be an agonist or antagonist, respectively, in cells containing ERβ or ERα where SRC-1a is the main coactivator. In addition, bisphenol A is likely to be most effective in cells containing ERβ where TIF2 is the main coactivator, but may be equally effective in cells containing either ERα or ERβ when SRC-1a is predominant. In other words, these results indicate that ligand-dependent differences in the ability of the ER to recruit coactivators may alter, in part, the receptors’ ability to potentiate gene expression in whole cells.

Transiently transfected HeLa cells were used to assess whether the differences in coactivator recruitment observed in the GST pull-down assays were also consistent with the ability of ERα and ERβ to transactivate an estrogen-responsive reporter gene construct with excess coactivator (SRC-1e or TIF2). The HeLa cell system brings together all the different components of ER action (ligand binding, receptor dimerization, DNA binding, coactivator recruitment, and gene transcription), and the activity of the receptor is measured by the strength of the luciferase (reporter gene) response. Within the context of the whole cell, it is impossible to conceal the receptor from the influence of other endogenous coactivators, corepressors, or...
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In all cases the response with 17β-estradiol was arbitrarily set at 100. Values were determined from data shown in Figs. 4 and 5.

| Chemical       | TIF2 RRA ERα | TIF2 RRA ERβ | SRC-1a RRA ERα | SRC-1a RRA ERβ | ERβ/ERα | ERβ/ERα |
|----------------|--------------|--------------|----------------|----------------|---------|---------|
| 17β-Estradiol  | 100.0        | 100.0        | 1.0            | 100.0          | 100.0   | 1.0     |
| Diethylstilbestrol | 11.0     | 60.0        | 5.5            | 5.0            | 50.0    | 10.0    |
| Genistein      | 0.005        | 60.0        | 12000.0        | 0.06           | 2.0     | 33.3    |
| PCB-OH         | None         | Slight (25%)| >1000.0        | None           | 0.05    | >1000.0 |
| 4-t-Octylphenol| 0.0002       | 0.2         | 1000.0         | 0.002          | 0.002   | 1.0     |
| Bisphenol A    | <0.0001      | 0.05        | >500.0         | 0.0003         | 0.0002  | 0.67    |

Interpretation of the cell line data was complicated by a detection-limit artifact in which the level of ERβ expression (in the absence of hormone and excess coactivator) was not resolved above the background level of the assay. Therefore, it was not possible to compare the behavior of ERα and ERβ in the presence and absence of excess coactivator. Potentiation of reporter gene activity in the presence of PCB-OH was consistently higher with ERβ than ERα in cells expressing SRC-1e. However, PCB-OH was able to potentiate reporter gene activity in the absence of excess coactivator with ERα, indicating that HeLa cells must contain other endogenous coactivators which can function in this case (Fig. 6). Genistein was more effective with ERβ than ERα with both SRC-1e and TIF2 (RRA ratio = 33 and 12,000, respectively) as predicted by the pull-down assays. Moreover, bisphenol A potentiated ERα and ERβ similarly with SRC-1e (RRA ratio = 0.67), but the response was greater with ERβ plus TIF2 (RRA ratio > 500). In all cases the magnitude of the reporter gene response was greater with ERα, whereas the relative increase in reporter gene activity was greater with ERβ. This suggests that factors other than the binding affinity of the ligand for the receptor and the ability of the receptor to recruit coactivators may also affect reporter gene activity. Nevertheless, given the increase in complexity between the binding assays and the whole cell system, there is a remarkable consistency in the results. Within the group of chemicals tested, we did not come across a ligand with ERα-selective coactivator recruitment, and therefore we were not able to compare this type of response profile in the transfection
A representative experiment are presented. Transiently transfected HeLa cells, carrying expression vectors for either Ga4-AF2a or Ga4-AF2b, were assessed for their ability to stimulate reporter gene expression (pGal4), TK.GL3 with E2 (10^{-8} M) or Bis (10^{-4} - 10^{-8} M) in the presence of excess SRC-1e (A) or TIF2 (B). Results (potentiation) are expressed as increase in the response above the base-line level in the absence of hormone. Mean values ± S.D. from a representative experiment are presented.

It was interesting to note that the two chemicals that showed the biggest differences between ERa and ERb coactivator recruitment were the isoflavone phytoestrogen (genistein) and a hydroxylated PCB metabolite (2,3,4,5-tetrachlorobiphenylo). The mixed agonistic-antagonistic effects of these chemical groups on estrogen-mediated processes in mammals and mammalian cells are well established (41–43). Many flavanoids have now been shown to competitively bind to ERa (15, 44) and induce reporter gene activity in transiently transfected MCF-7 cells and yeast containing E2-responsive reporter constructs (45, 46). However, the same flavanoids are inactive in hormone-dependent cell proliferation assays (MCF-7), and may inhibit both the proliferative activity of E2 in co-treated MCF-7 cells and E2-induced gains in uterine weight in immature rats (45, 47). One possible explanation for this disparity is that different ligand-induced conformational changes in the receptor may enable ERa (the predominant ER form in the mammary gland and uterus) to activate gene expression on certain promoters, but not on others. Therefore, the much reported promoter-context specific action of ER action may be a consequence of the type of coactivators present, and the conformational change of the ER induced by the ligand. The fact that genistein had a significantly higher binding affinity and relative recruitment ability for SRC-1a and TIF2 with ERb versus ERa was intriguing, given the reported high expression of ERb in the secretory epithelial cells of the prostate (15), and the putative role these compounds play in preventing prostate cancer (48).

In utero and lactational exposure to PCBs (or commercial mixtures called “Arochlors”) is associated with persistent neurobehavioral, reproductive, and endocrine alterations (reviewed in Ref. 49), which are species-, age-, and congener-specific. Metabolism of PCBs by humans and rodents results in the formation of hydroxylated PCBs, many of which have been shown to be estrogenic in MCF-7 cells and transiently transfected HeLa cells (50). However, few, if any, studies have investigated the estrogenic and/or antiestrogenic activity of individual PCB congeners on the pituitary-hypothalamic axis and uterus in vivo, and therefore it is currently difficult to speculate whether the complex tissue-dependent effects of Arochlors are mediated by one or more specific congeners, which have selective ERa/ERb agonistic-antagonistic effects. However, the fact that ERa is the predominant form expressed in the stromal and epithelial cells of the endometrium (uterus), and ERb is expressed in high amounts in the paraventricular and supraoptic nucleus of the hypothalamus (4) implies that the predominant ER isoform present may determine, in part, the type of tissue response observed following PCB exposure, i.e. the heterogeneity of estrogen receptor distribution, and the predominant types of coactivators present, may contribute to the diversity of tissue responses to estrogenic chemicals. However, the true significance of these findings may only become apparent when the functional roles of ERs and ERb with the various receptor-interacting proteins are known. These roles may become clearer when dominant negative versions of receptor-interacting proteins are analyzed, or when knockout animals are generated.

Fig. 8. Potentiation of reporter gene activity by ERa-AF2 and ERb-AF2 with E2 and Bis in the presence of excess coactivator.

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