

Phytoestrogens and Their Human Metabolites Show Distinct Agonistic and Antagonistic Properties on Estrogen Receptor α (ER α) and ER β in Human Cells

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Phytoestrogens exert pleiotropic effects on cellular signaling and show some beneficial effects on estrogen-dependent diseases. However, due to activation/inhibition of the estrogen receptors ER α or ER β , these compounds may induce or inhibit estrogen action and, therefore, have the potential to disrupt estrogen signaling. We performed a comprehensive analysis and potency comparison of phytoestrogens and their human metabolites for ER binding, induction/suppression of ER α and ER β transactivation, and coactivator recruitment in human cells. The soy-derived genistein, coumestrol, and equol displayed a preference for transactivation of ER β compared to ER α and were 10- to 100-fold less potent than diethylstilbestrol. In contrast, zearalenone was the most potent phytoestrogen tested and activated preferentially ER α . All other phytoestrogens tested, including resveratrol and human metabolites of daidzein and enterolactone, were weak ER agonists. Interestingly, the daidzein metabolites 3',4',7-isoflavone and 4',6,7-isoflavone were superagonists on ER α and ER β . All phytoestrogens tested showed reduced potencies to activate ER α and ER β compared to diethylstilbestrol on the estrogen-responsive C3 promoter compared to a consensus estrogen response element indicating a degree of promoter dependency. Zearalenone and resveratrol were antagonistic on both ER α and ER β at high doses. The phytoestrogens enhanced preferentially recruitment of GRIP1 to ER α similar to 17 β -estradiol. In contrast, for ER β no distinct preference for one coactivator (GRIP1 or SRC-1) was apparent and the overall coactivator association was less pronounced than for ER α . Due to their abundance and (anti)-estrogenic potencies, the soy-derived isoflavones, coumestrol, resveratrol, and zearalenone would appear to have the potential for effectively functioning as endocrine disruptors.

Key Words: ER α ; ER β ; xenoestrogen; endocrine disruptors; Ishikawa; coactivator recruitment.

The estrogenic effects of a variety of structurally diverse plant-derived compounds are mediated through the estrogen receptors ER α and ER β , which function as ligand-inducible

transcription factors for genes involved in cell growth, proliferation, and differentiation (Hall *et al.*, 2001; Mueller and Korach, 2001b). Due to activation of the ER, these compounds are referred to as phytoestrogens and have the potential to disrupt estrogenic signaling. *In vitro* studies indicated that ER α and ER β display marked differences in binding affinity and activation by some natural and synthetic ER ligands (Kuiper *et al.*, 1997, 1998). Interestingly, although ER β shows lower binding affinity for and activation by endogenous estrogens, some xenoestrogens preferentially bind and activate ER β (Kuiper *et al.*, 1998). Although epidemiological data indicated that several phytoestrogens like the soy isoflavones may have protective effects on estrogen-dependent malignancies (Adlercreutz, 1995), data on disruption of ER function, adverse effects observed in rodents (Jefferson and Newbold, 2000), and the eventually high doses of dietary phytoestrogens (Bingham *et al.*, 1998) warrant a more thorough analysis of the potential dysregulation of ER action by phytoestrogens.

Most phytoestrogens exert pleiotropic effects involving kinase inhibition, cell cycle regulation, and antioxidative properties that are likely to contribute to the beneficial effects of phytoestrogens (Basly *et al.*, 2000; Cappelletti *et al.*, 2000). Nevertheless, estrogenic and/or antiestrogenic activities of phytoestrogens like resveratrol and genistein may reduce but also stimulate estrogen-dependent tumor growth depending on dose and timing of exposure (Allred *et al.*, 2001; Bhat *et al.*, 2001; Cotroneo *et al.*, 2002).

So, we have analyzed a selection of phytoestrogens (Fig. 1), with known or suspected estrogenic action (reviewed in Cassidy *et al.*, 2000; Mazur and Adlercreutz, 2000; Mueller, 2002), in a series of cellular assays for dose-dependent agonistic and antagonistic activity on ER α and ER β . One major drawback of cellular assays is that the cell lines used are incapable of metabolizing xenobiotics. Therefore, we have evaluated some of the major human metabolites of enterolactone and daidzein for their estrogenic activities (Fig. 1; Jacobs *et al.*, 1999; Niemeyer *et al.*, 2000). Several reports have been published that characterized selected features of phytoestrogen action

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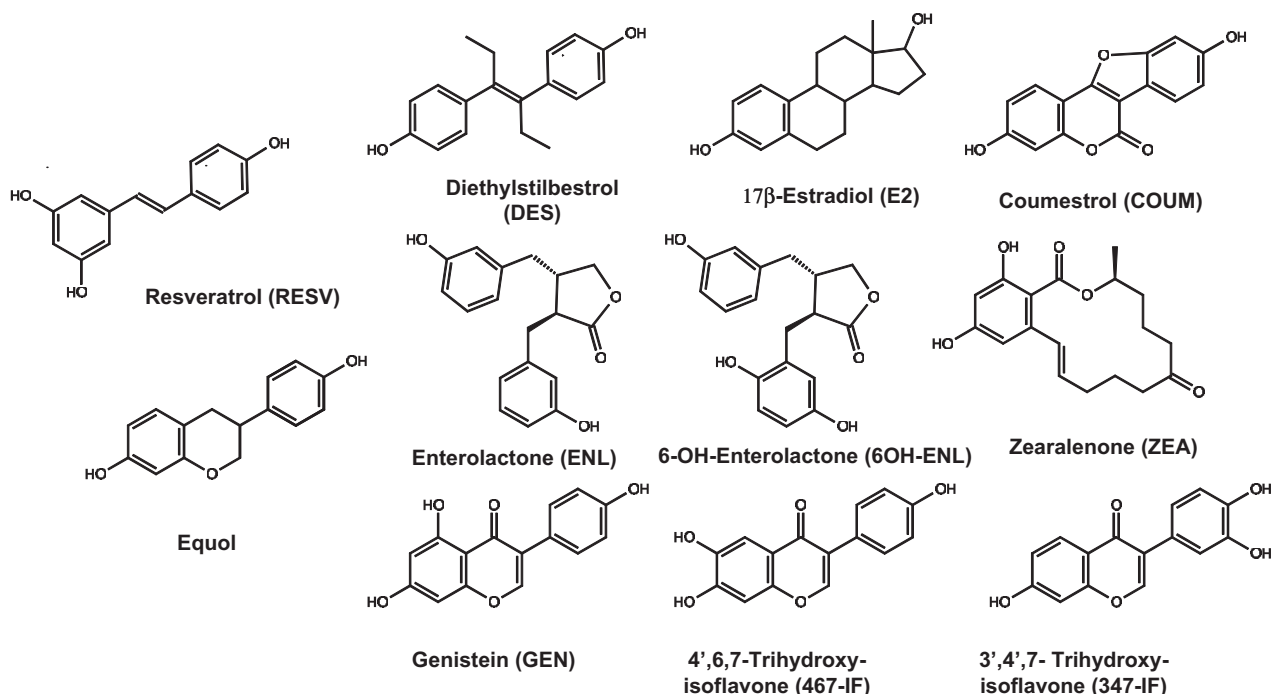


FIG. 1. Chemical structures of the investigated compounds.

including receptor binding, ER transactivation, or target gene expression (reviewed in Mueller, 2002). However, a comprehensive analysis of the potencies of several phytoestrogens in one comparable system for both ER α and ER β activities is lacking. Thus, we have employed a human endometrial Ishikawa cell line that stably expresses human ER α or ER β (Mueller *et al.*, 2003a) to assess the estrogenic and antiestrogenic potencies of the selected phytoestrogens (Fig. 1). Furthermore, binding affinities for ER α and ER β were analyzed. To elucidate the mechanism of ER activation or inhibition by phytoestrogens in more detail, we evaluated the recruitment of coactivators to ER α and ER β by selected phytoestrogens in mammalian two-hybrid assays. By using this approach, we were able to rank and characterize the analyzed phytoestrogens for their estrogenic and/or antiestrogenic properties in human cells.

MATERIALS AND METHODS

Materials and biochemicals. Media, supplements, enzymes, and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Steroid-deprived, dextran-coated, charcoal-treated fetal bovine serum (DCC/FBS) was from Hyclone (lot AKD11642A, Perbio Science, Bonn, Germany). ICI 182,780 (ICI) was from Tocris (Bristol, UK). Test compounds and phytoestrogens were purchased from Sigma Chemical Co. or Fluka Chemical Corp. (Ronkonkoma, NY) and were of the highest purity available. Enterolactone (ENL, purity > 99%) and its human metabolite 6-hydroxy-enterolactone (6OH-ENL, purity > 99%) were synthesized and purified as described previously (Jacobs and Metzler, 1999; Jacobs *et al.*, 1999; Niemeyer *et al.*, 2000). The human daidzein metabolites 4',6,7-trihydroxy-isoflavone (467-IF, purity 98%) and 3',4',7-trihydroxy-isoflavone (347-IF, purity > 98%) were from Fluka Chemical Corp.

Cell culture. Human endometrial Ishikawa cells with stable expression of human ER α (Ishikawa-hER α) or human ER β (Ishikawa-hER β ; Mueller *et al.*, 2003a) were cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 10% DCC/FBS, as described previously (Mueller *et al.*, 2003a). Human hepatoma HepG2 cells were maintained in DMEM/F12 supplemented with 10% FBS, sodium pyruvate, and antibiotics. Cells were cultured at 37°C/5% CO₂ in air in a humidified atmosphere.

Ligand-binding studies by fluorescence polarization. Ligand-binding affinities to purified human ER α and ER β were determined by competition binding of the test compounds against fluorescein-labeled estradiol (ES2) using an ER-screening kit according to the manufacturer's protocol (Panvera, Göttingen, Germany). Recombinant human ER α or ER β (10 nM) were incubated with the test compounds for 2 h in the presence of 1 nM ES2 (Panvera). Fluorescence polarization was measured using a Polarion multiwell plate reader (Tecan, Crailsheim, Germany). Ligand concentration yielding 50% inhibition of binding of fluorescently labeled ES2 to ER (IC₅₀) was derived by nonlinear curve-fitting using ORIGIN software (Microcal Software, Northampton, MA) from competition binding curves. The IC₅₀ were determined from at least three independent experiments and are given as mean \pm standard deviation.

Transient transfection and transactivation assay. Ishikawa-hER α and Ishikawa-hER β cells were seeded on 24-well plates 15 h prior to transfection in phenol red-free DMEM/F12 supplemented with 10% DCC/FBS. The plasmids were transfected in phenol red-free DMEM/F12 supplemented with 5% DCC/FBS using Fugene 6 (Roche, Mannheim, Germany), according to the manufacturer's protocol. Each well received 0.5 μ g of reporter plasmid and 0.01 μ g pRL-CMV (*Renilla* luciferase for normalization; Promega, Madison, WI). A firefly luciferase reporter driven by three copies of the vitellogenin estrogen response element (3 \times ERE-Luc) and a reporter containing the human complement 3 gene (C3) promoter (reporter kindly provided by D. P. McDonnell, Duke University, Durham, NC) were used to measure ER transcriptional activity. Test compounds (final concentration of vehicle ethanol 1% v/v) in 200 μ l phenol red-free DMEM/F12 supplemented with 10% DCC/FBS were added to the cells after transfection and incubated for 22 h. Luciferase assays were performed using

the dual-luciferase reporter assay system according to the manufacturer's protocol (Promega). Each value was normalized to the *Renilla* luciferase control and each data point obtained represents the average of duplicate determinations. All experiments were repeated at least three times. EC₅₀ values (ligand concentration yielding half-maximal activation) or IC₅₀ values (ligand concentration yielding half-maximal inhibition of DES-mediated activity) were derived by nonlinear curve-fitting using ORIGIN software (Microcal Software) from transactivation curves and are given as mean ± standard deviation of at least three independent experiments.

Mammalian two-hybrid assays. For mammalian two-hybrid assays, HepG2 cells were plated in 24-well plates 15 h prior to transfection. The plasmids were transfected in phenol red-free DMEM/F12 supplemented with 5% DCC/FBS using Fugene 6 (Roche), as described previously (Mueller *et al.*, 2003a). In standard transfections, 0.5 µg of reporter 5' × -Gal4-TATA-Luc, containing five binding sites for the yeast Gal4 transcription factor, 0.09 µg of receptor (either pVP16-hERα [GenBank accession M12674] or pVP16-hERβ [GenBank accession AF051427, "short" form of ERβ]), 0.5 µg of Gal4 DNA binding domain fused to the nuclear receptor interaction domain (NR-box) of each coactivator (pM-SRC-1 [NR box] or pM-GRIP1 [NR box]; plasmids kindly provided by D. P. McDonnell), and 0.01 µg of the pRL-CMV *Renilla* luciferase normalization vector were used for each well. Test compounds in 200 µl phenol red-free DMEM/F12 supplemented with 10% DCC/FBS were then added to the cells and incubated for 22 h. Luciferase assays were performed using the dual-luciferase reporter assay system according to the manufacturer's protocol. Each value was normalized to the *Renilla* luciferase control, and each data point generated is the average with range of duplicate determinations. All experiments were performed three times.

RESULTS

Phytoestrogens Showed Distinct Binding Affinities to Human ERα and ERβ

Binding of a ligand to the ER is the first determinant for its potential to act as an ER agonist or antagonist. Therefore, we analyzed the binding affinities of the following phytoestrogens: coumestrol (COUM); the isoflavone genistein (GEN); the human daidzein metabolites equol, 4',6,7-trihydroxy-isoflavone (467-IF), and 3',4',7-trihydroxy-isoflavone (347-IF); the stilbene resveratrol (RESV); the resorcylic acid lactone zearalene (ZEA); and the lignans enterolactone (ENL) and its human metabolite 6-hydroxy-enterolactone (6OH-ENL; Fig. 1). Competition binding studies were performed with purified human ERα or ERβ and fluorescein-labeled E2 (Mueller *et al.*, 2003b). A ranking of the relative binding affinities (RBA) confirmed that GEN, COUM, ZEA, and equol have the highest binding affinities to ERα and ERβ and that GEN, COUM, and equol showed a distinct preference for ERβ (Table 1; Kuiper *et al.*, 1998; Mueller, 2002). The other phytoestrogens tested showed rather weak binding affinities compared to the potent synthetic estrogen diethylstilbestrol (DES) and the endogenous estrogen 17β-estradiol (E2; Table 1).

Phytoestrogens Exerted Distinct Agonistic and/or Antagonistic Activity on Human ERα and ERβ

Binding affinity to ER has been used frequently as a surrogate marker for estrogenicity. However, binding to the ER does not

TABLE 1
Relative Binding Affinities (RBA) of Phytoestrogens for Human ERα and ERβ

Ligand	ERα IC ₅₀ ^a	RBA ^b	ERβ IC ₅₀	RBA
DES	4.6 ± 1.0 nM	100	4.6 ± 1.5 nM	100
E2	4.3 ± 1.1 nM	107	5.7 ± 0.7 nM	82
COUM	38 ± 15 nM	12	6.0 ± 3.6 nM	77
ZEA	56 ± 15 nM	8	41 ± 18 nM	11
GEN	0.3 ± 0.2 µM	1	15 ± 7.0 nM	31
Equol	1.5 ± 0.5 µM	0.3	0.2 ± 0.01 µM	3
467-IF	3.1 ± 0.5 µM	0.1	16 ± 5.2 µM	0.03
6OH-ENL	5.6 ± 0.9 µM	0.08	51 ± 44 µM	0.009
ENL	6.7 ± 4.3 µM	0.07	39 ± 22 µM	0.01
RESV	7.7 ± 2.3 µM	0.06	29 ± 20 µM	0.02
347-IF	23 ± 7.3 µM	0.02	4.9 ± 3.1 µM	0.09

^aIC₅₀ values (ligand concentration yielding 50% inhibition of binding of fluorescein-labeled E2 to ER) were derived by nonlinear curve-fitting from competition binding curves and are given as mean ± standard deviation of at least three independent experiments.

^bRBA values were calculated by $100 \times \text{IC}_{50}(\text{DES})/\text{IC}_{50}(\text{test compound})$. DES was set to 100.

necessarily result in agonistic activity and may lead to antagonistic activity on ERα or ERβ. Previously, we have performed transactivation assays in human endometrial Ishikawa cells that stably express human ERα (Ishikawa-hERα) or ERβ (Ishikawa-hERβ; Mueller *et al.*, 2003a). The parental Ishikawa cells (Ignar-Trowbridge *et al.*, 1995) lack endogenously active ERα and ERβ and the stably transfected cell lines offer the advantage of constitutive expression of either human ERα or ERβ (Mueller *et al.*, 2003a). It is by now well established that the promoter sequence of estrogen target genes can determine the activity of liganded ER (Hall and Korach, 2002; Hall *et al.*, 2002; Klinge, 2001). So, we analyzed the activity of phytoestrogens on a reporter with three copies of the vitellogenin A consensus ERE (3 × ERE; Norris *et al.*, 1997) and on the estrogen-responsive C3 promoter that contains several nonconsensus EREs (Norris *et al.*, 1996).

The dose-response transactivation curves of the tested phytoestrogens on the 3 × ERE reporter are shown in Figure 2. For comparison of the estrogenic potencies of phytoestrogens with that of DES and E2, we calculated the EC₅₀ values for ER transactivation. Also, the efficacy, i.e., the maximum-fold induction over control observed, was also determined (Fig. 3). The overall ranking of the potency was consistent with the observed binding affinities (Table 1). ZEA and COUM were the most potent phytoestrogens on ERα, and COUM, GEN, and equol preferentially activated ERβ (Fig. 3) in accordance with published reports (reviewed in Mueller, 2002). In contrast, ZEA had higher potencies on ERα than on ERβ (Fig. 3). RESV and ENL showed very low potency to induce ERα or ERβ activity; 6OH-ENL and the daidzein metabolites 347-IF and 467-IF displayed the lowest potencies to activate ERα or ERβ (Fig. 3).

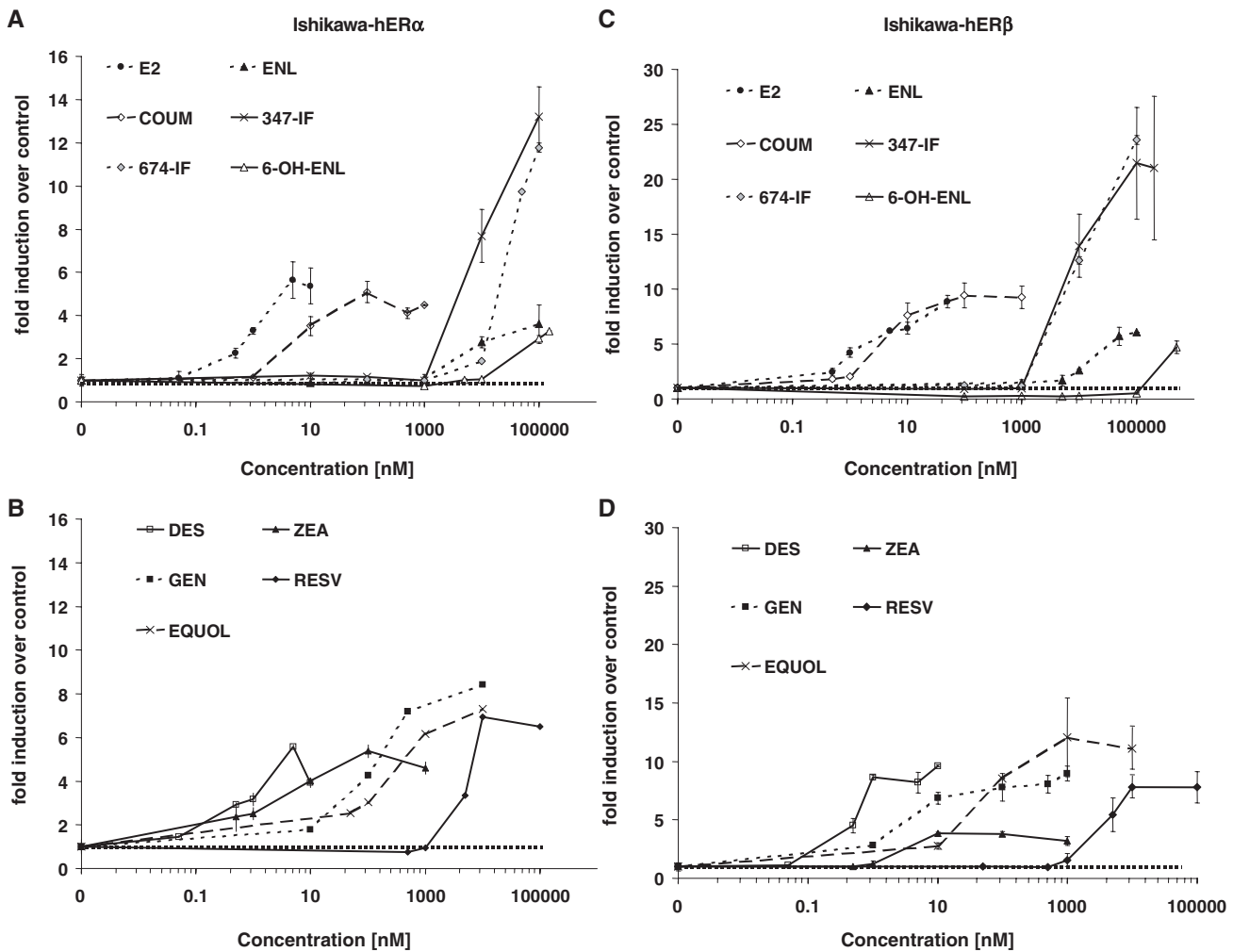


FIG. 2. Transactivation in Ishikawa cells stably expressing human ER α (Ishikawa-hER α) or ER β (Ishikawa-hER β): Agonistic activity of phytoestrogens. (A and B) Ishikawa-hER α or (C and D) Ishikawa-hER β cells were transiently transfected with $3 \times$ ERE-Luc and pRL-CMV. Following transfection, cells were treated with each compound at the indicated concentrations for 22 h. Each value was normalized to the internal luciferase control and results are expressed as the fold induction compared to the solvent control. The dotted line indicates the value of the solvent control (set to 1). Each data point represents the average with range of duplicate determinations. Experiments were performed at least three times with reproducible results.

Comparison of the activities on the consensus $3 \times$ ERE with the C3 promoter revealed that the potency of the phytoestrogens was in general lower on the C3 promoter (Fig. 3); however, the ranking was not affected with one exception. ZEA, rather potent to induce ER β activity on the $3 \times$ ERE, transactivated ER β only 2-fold compared to untreated control on the C3 promoter. Therefore, no EC₅₀ value could be calculated (Fig. 3C).

Another measure of estrogenic activity is the efficacy. ZEA, COUM, GEN, equol, and RESV had efficacies that were comparable to DES and E2 (Fig. 3). Therefore, these compounds were considered full agonists. ENL and 6OH-ENL showed markedly lower efficacies than DES and were considered partial agonists (Fig. 3). More interestingly, the daidzein metabolites 347-IF and 467-IF showed distinctively higher-fold induction than DES or E2 (Figs. 2 and 3). These high efficacies were observed at doses of 10 μ M and higher only. These

compounds resembled superagonists but with very weak potencies.

Next to the agonistic activity, the antagonistic activity on ER is important for the characterization of endocrine-active compounds. All phytoestrogens (Fig. 1) were tested for their potency to inhibit DES-induced, ER-mediated activity in Ishikawa-hER α and Ishikawa-hER β . As expected, the partial agonists ENL and 6OH-ENL acted as partial antagonists (20–40% reduction of DES activity, data not shown). Distinct antagonistic activities were observed for RESV and ZEA only (Fig. 4). All other phytoestrogens tested did not inhibit ER activity at doses up to 100 μ M (data not shown). RESV has been identified as a mixed ER α agonist/antagonist (Bhat *et al.*, 2001; Bowers *et al.*, 2000). We analyzed in more detail its antagonistic properties with regard to ER α and ER β . In contrast to ZEA that showed dose-dependent antagonistic activity on ER α and ER β , RESV

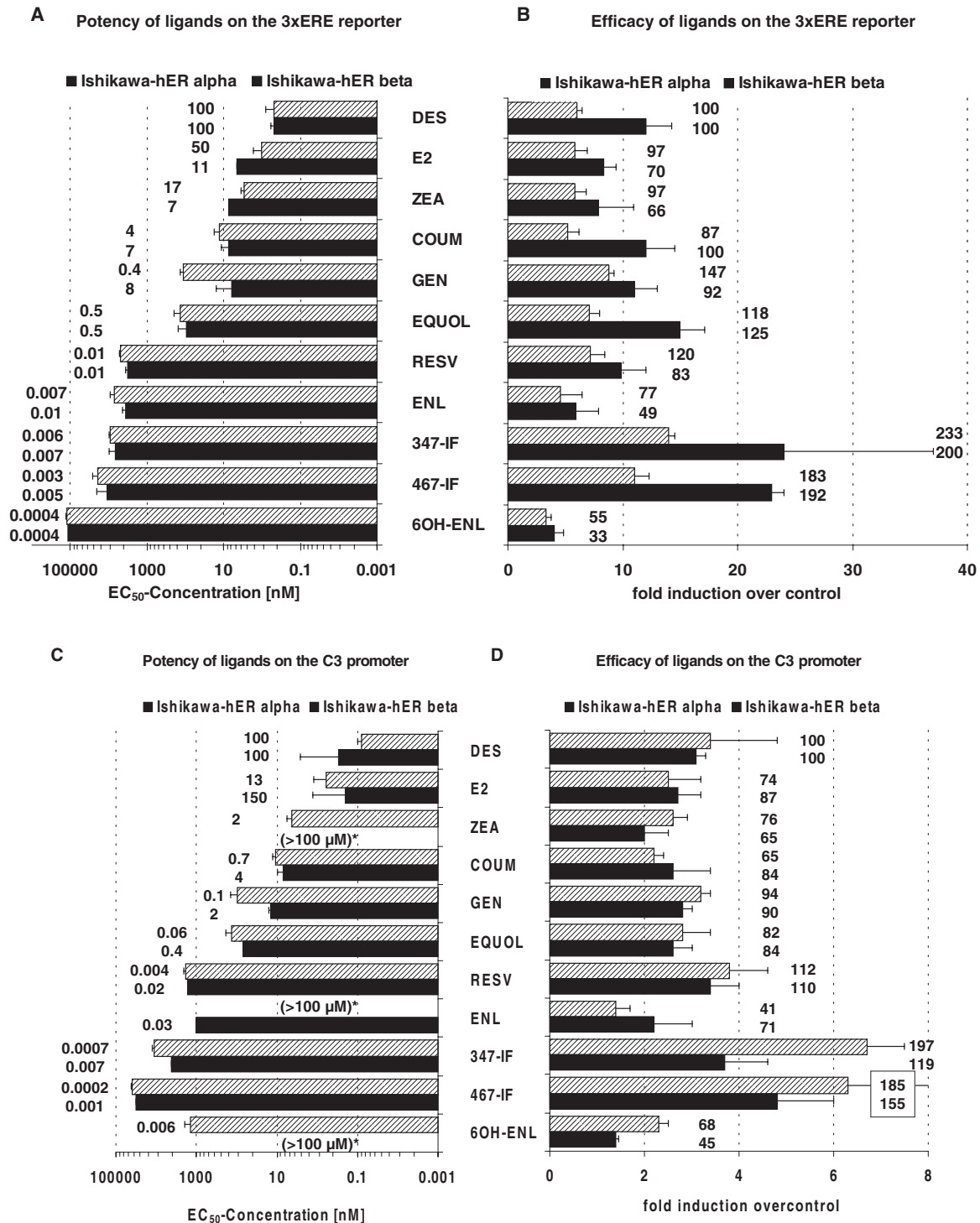


FIG. 3. Transactivational potencies and efficacies of DES, E2, and phytoestrogens in Ishikawa cells stably expressing human ER α (Ishikawa-hER α) or ER β (Ishikawa-hER β). Transcriptional activity was measured on reporter vectors containing three copies of (A and B) the consensus ERE (3 \times ERE) or (C and D) the human C3 promoter. Relative potency was calculated by $100 \times EC_{50}(\text{DES})/EC_{50}(\text{test compound})$ and is indicated next to each column. DES was set to 100. EC_{50} values (ligand concentration yielding half-maximal activation) were derived by nonlinear curve-fitting from transactivation curves obtained in Ishikawa-hER α or Ishikawa-hER β cells (e.g., Fig. 2) and are given as mean \pm standard deviation of at least three independent experiments. Please note (A and C) the logarithmic scale of EC_{50} values. Relative efficacy was calculated by $100 \times \text{efficacy}(\text{DES})/\text{efficacy}(\text{test compound})$. DES was set to 100. (B and D) Efficacies (maximum fold induction over control) were determined from transactivation curves obtained in Ishikawa-hER α or Ishikawa-hER β cells (e.g., Fig. 2) and are given as mean \pm standard deviation of at least three independent experiments. * EC_{50} value could not be determined; therefore, the highest dose tested is given in parentheses.

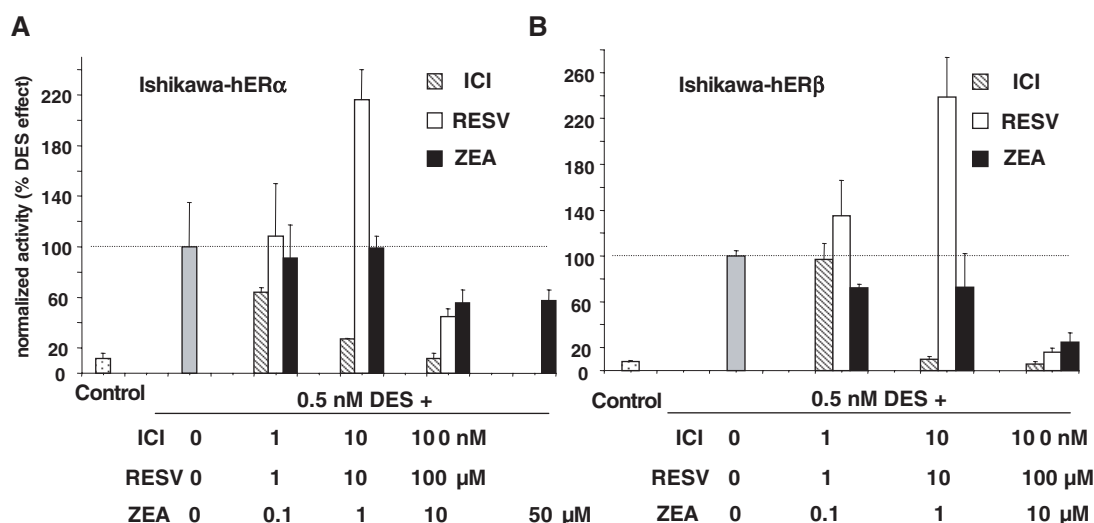


FIG. 4. Transactivation in Ishikawa cells stably expressing human ER α (Ishikawa-hER α) or ER β (Ishikawa-hER β): Antagonistic activity of phytoestrogens. (A) Ishikawa-hER α or (B) Ishikawa-hER β cells were transiently transfected with 3 \times ERE-Luc and pRL-CMV. Following transfection, cells were treated with vehicle (control, dotted columns) or 0.5 nM diethylstilbestrol (DES, grey columns) and ICI 182,780 (ICI, striped columns), resveratrol (RESV, open columns), or zearalenone (ZEA, solid columns) as indicated for 22 h. Each value was normalized to the internal luciferase control. The dotted line indicates the relative value of the positive control DES (set to 100%). Each data point represents the average with range of duplicate determinations. Experiments were performed at least three times with reproducible results.

TABLE 2

Comparison of ER-Antagonistic Activities of ICI 182,780 (ICI), Resveratrol (RESV), and Zearalenone (ZEA) in Ishikawa Cells Stably Expressing Human ER α (Ishikawa-hER α) or ER β (Ishikawa-hER β)^a

Ligand	Ishikawa-hER α	Ishikawa-hER β
ICI	5.3 \pm 3.7 nM ^b	2.8 \pm 0.4 nM
RESV	33 \pm 11 μ M	35 \pm 12 μ M
ZEA	8.1 \pm 4.3 μ M	2.8 \pm 1.4 μ M

^aInhibition of transcriptional activity by test compounds was measured in the presence of 0.5 nM DES on the consensus ERE (3 \times ERE) reporter vector in Ishikawa-hER α and Ishikawa-hER β .

^bIC₅₀ values (ligand concentration yielding 50% inhibition of DES-induced activity) were derived by nonlinear curve-fitting from transactivation curves obtained in Ishikawa-hER α or Ishikawa-hER β cells (e.g., Fig. 4) and are given as mean \pm standard deviation of at least three independent experiments.

displayed an inverted U-shaped dose response (Fig. 4). At low doses, RESV increased the DES-induced activity of ER α and ER β , but at high doses it inhibited activity of ER α and ER β (Fig. 4). We then compared the antiestrogenic potencies of RESV and ZEA with the pure antiestrogen ICI (Wakeling and Bowler, 1992). The IC₅₀ values for the inhibition of DES-induced activity are shown in Table 2. ZEA showed a higher antiestrogenic potency than RESV; both RESV and ZEA showed similar potencies on ER α and ER β in the μ M range, whereas ICI was antiestrogenic at nM doses (Table 2).

Phytoestrogens Exhibited a Distinct Enhancement and/or Repression of Coactivator Recruitment to ER α and ER β

Activation of the ER depends on the proper recruitment of coactivators that facilitate the transcription of ER target genes (Tremblay and Giguere, 2002). Recruitment of coactivators may, therefore, also determine the ER subtype-specific activation by phytoestrogens (Hall and Korach, 2002). We analyzed the recruitment of two major coactivators, GRIP1 also known as TIF2 (Voegel *et al.*, 1996) and SRC-1 (Onate *et al.*, 1995), that interact with ER α and ER β and are able to facilitate ER transactivation in HepG2 cells (Mueller *et al.*, 2003a). Firstly, we performed dose-response curves with the compounds tested in two-hybrid assays in human HepG2 cells. All agonists tested, DES, E2, ZEA, COUM, GEN, and RESV, induced a dose-dependent increase in the recruitment of both GRIP1 and SRC-1 to ER α and ER β (data not shown). The daidzein metabolites 347-IF and 467-IF displayed distinct coactivator recruitment to ER α and ER β at doses of 10 μ M and higher, which is in agreement with the transactivation data (Figs. 2 and 3). For comparison of the selected phytoestrogens, we chose the doses for each compound that elicited the maximum response in coactivator recruitment (Fig. 5). ER α preferentially associated with GRIP1 compared to SRC-1 in the presence of the potent estrogens E2 and DES. ZEA, a potent ER α agonist (Fig. 3), induced strong association of GRIP1 to ER α similar to E2 and DES (Fig. 5A). ER α but not ER β showed preferential association with GRIP1 over SRC-1 in the presence of the ER agonists COUM, GEN, and RESV, whereas the superagonists 347-IF and 467-IF showed no differences and induced similar

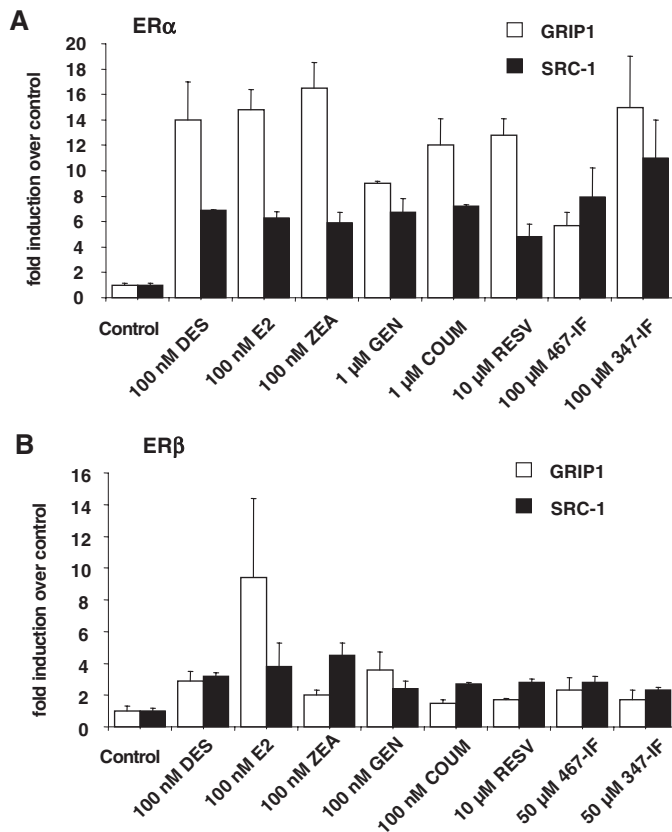


FIG. 5. Phytoestrogens showed distinct abilities to recruit coactivators to ER α and ER β . Mammalian two-hybrid assays were used to quantify the ligand-induced interaction of human ER α and ER β with the coactivators GRIP1 and SRC-1. For these experiments, constructs containing the GRIP1 and SRC-1 receptor interaction domains (NR-box) fused to the Gal4 transcription factor DNA-binding domain (pM-GRIP1 and pM-SRC-1) were used together with constructs containing either the human ER α or ER β cDNA fused in frame to the VP16 activation domain (pVP16-hER α and pVP16-hER β). HepG2 cells were transiently transfected with the 5 \times -Gal4-TATA-Luc reporter, the pRL-CMV normalization plasmid, and either pM-GRIP1 (GRIP1, open columns) or pM-SRC-1 (SRC-1, solid columns) together with (A) pVP16-hER α or (B) pVP16-hER β . Following transfection, cells were treated with vehicle (control) or test compound at the indicated concentration. After 22 h, cells were harvested and dual luciferase assays were performed. Each value was normalized to the internal luciferase control. Shown is a representative experiment with each data point being the average with range of duplicate determinations. Experiments were performed three times with consistent results.

association of SRC-1 and GRIP1 to ER α and ER β (Fig. 5). For ER β , no distinct preference for GRIP1 or SRC-1 was apparent (Fig. 5B).

Since RESV and ZEA were antiestrogenic in Ishikawa-hER α and Ishikawa-hER β (Fig. 4 and Table 2), we analyzed whether RESV or ZEA were able to repress coactivator recruitment induced by DES (Figs. 6 and 7). As positive control, we tested the pure ER antagonist ICI that completely repressed coactivator recruitment induced by DES (data not shown). RESV also repressed recruitment of either GRIP1 or SRC-1 to ER α and

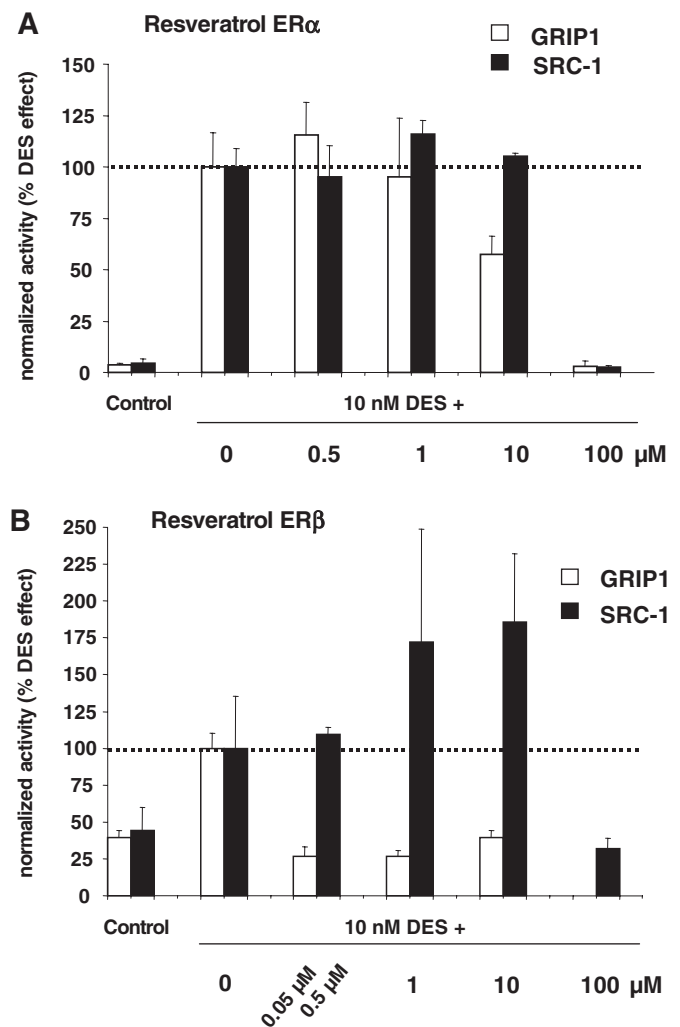


FIG. 6. Inhibition of coactivator recruitment to ER by resveratrol (RESV). Mammalian two-hybrid assays were used to quantify the interaction of human ER α with the coactivators GRIP1 and SRC-1 in the presence of RESV. For these experiments, constructs containing the GRIP1 and SRC-1 receptor interaction domains (NR-box) fused to the Gal4 transcription factor DNA-binding domain (pM-GRIP1 and pM-SRC-1) were used together with constructs containing either the human ER α or ER β cDNA fused in frame to the VP16 activation domain (pVP16-hER α or pVP16-hER β). HepG2 cells were transiently transfected with the 5 \times -Gal4-TATA-Luc reporter, the pRL-CMV normalization plasmid, and either pM-GRIP1 (GRIP1, open columns) or pM-SRC-1 (SRC-1, solid columns) together with (A) pVP16-hER α or (B) pVP16-hER β . Following transfection, cells were treated with vehicle (control) or 10 nM diethylstilbestrol (DES) and RESV as indicated for 22 h. After 22 h, cells were harvested and dual luciferase assays were performed. The dotted line indicates the relative value of the positive control DES (set to 100%). Each value was normalized to the internal luciferase control. Shown is a representative experiment with each data point being the average with range of duplicate determinations. Experiments were repeated three times with consistent results.

ER β at high doses (10–100 μ M; Fig. 6). Recruitment of both GRIP1 and SRC-1 to ER α showed no distinct inverted U-shaped dose response when cells were treated with DES together with RESV (Fig. 6). The characteristic inverted U-shaped dose

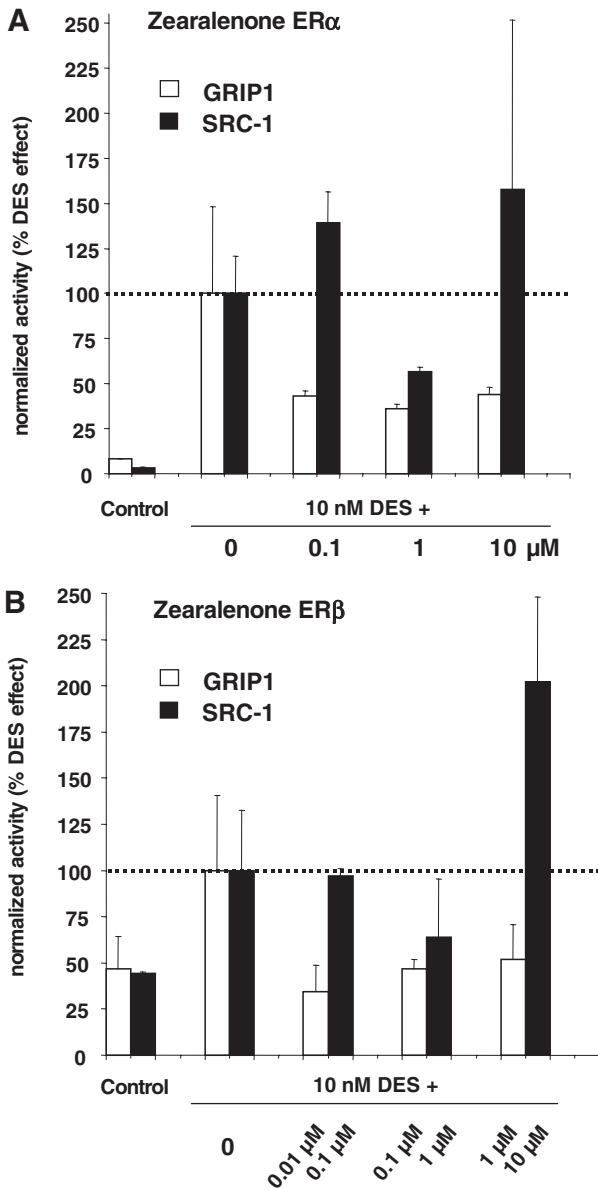


FIG. 7. Inhibition of coactivator recruitment to ER by zearalenone (ZEA). Mammalian two-hybrid assays were used to quantify the interaction of human ER α with the coactivators GRIP1 and SRC-1 in the presence of ZEA. For these experiments, constructs containing the GRIP1 and SRC-1 receptor interaction domains (NR-box) fused to the Gal4 transcription factor DNA-binding domain (pM-GRIP1 and pM-SRC-1) were used together with constructs containing either the human ER α or ER β cDNA fused in frame to the VP16 activation domain (pVP16-hER α or pVP16-hER β). HepG2 cells were transiently transfected with the 5 \times -Gal4-TATA-Luc reporter, the pRL-CMV normalization plasmid, and either pM-GRIP1 (GRIP1, open columns) or pM-SRC-1 (SRC-1, solid columns) together with (A) pVP16-hER α or (B) pVP16-hER β . Following transfection, cells were treated with vehicle (control) or 10 nM diethylstilbestrol (DES) and ZEA as indicated for 22 h. After 22 h, cells were harvested and dual luciferase assays were performed. The dotted line indicates the relative value of the positive control DES (set to 100%). Each value was normalized to the internal luciferase control. Shown is a representative experiment with each data point being the average with range of duplicate determinations. Experiments were repeated three times with consistent results.

response, as observed in Figure 4, was apparent for the recruitment of SRC-1 but not GRIP1 to ER β only (Fig. 6B). ZEA reduced recruitment of GRIP1 to ER α and ER β in a dose-dependent fashion (Fig. 7), in agreement with the transactivation data (Fig. 4). Interestingly, recruitment of SRC-1 to ER α and ER β was not consistently impaired by ZEA (Fig. 7). These results indicated that phytoestrogens are able to alter the recruitment of coregulators depending on the type of coactivator and ER subtype.

DISCUSSION

Most phytoestrogens exert pleiotropic effects on cellular signaling and show some beneficial effects on estrogen-dependent diseases (Adlercreutz, 1995). However, these compounds can also promote tumor growth (Hilakivi-Clarke *et al.*, 1999a; Newbold *et al.*, 2001) and cause developmentally adverse effects (Delclos *et al.*, 2001; Jefferson and Newbold, 2000). Although the beneficial effects, such as the prevention of mammary carcinomas by genistein in rodents (Murrill *et al.*, 1996), have been attributed to the antiestrogenic effects, experimental evidence suggests that estrogenic effects of isoflavones and potent estrogens during prepubertal development may prevent mammary tumors (Cotroneo *et al.*, 2002; Grubbs *et al.*, 1985; Hilakivi-Clarke *et al.*, 1999b; Murrill *et al.*, 1996). However, these estrogenic effects are likely to turn into a detrimental response, i.e., tumor promotion, during later stages of development. In contrast, antiestrogenic compounds can prevent or reduce estrogen-dependent tumor growth independent of timing of exposure (Bhat *et al.*, 2001). Thus, these results warrant a cautious evaluation of the use of phytoestrogens to prevent estrogen-dependent diseases and require a thorough analysis of the estrogenic and antiestrogenic properties of these compounds.

In an attempt to provide a comprehensive analysis and potency comparison of the (anti-) estrogenicity of phytoestrogens, we evaluated several abundant phytoestrogens for ER binding, activation/inhibition, and coactivator recruitment in human cells. For this, we used a cell line with defined expression of either human ER α or ER β (Mueller *et al.*, 2003a). Since this cell line lacks activity to metabolize xenobiotics, we also analyzed some major human metabolites. The physiological effects of estrogens are mediated predominantly by ER α (Couse and Korach, 1999; Mueller and Korach, 2001a), whereas ER β may counteract ER α action (Hall and McDonnell, 1999; Liu *et al.*, 2002) and has an important role at least in ovarian development (Couse and Korach 1999; Couse *et al.*, 1999). Therefore, it is crucial for the evaluation of the (anti-)estrogenicity of phytoestrogens to analyze both ER α and ER β . Kuiper and colleagues were the first ones to compare several xenoestrogens for ER α versus ER β binding and activity (Kuiper *et al.*, 1998). However, no study has yet compared the potencies and characteristics of various phytoestrogens and their metabolites for ER α and ER β activity in a comprehensive manner.

TABLE 3
Compilation of Characteristic Estrogenic and Antiestrogenic Activities^a of the Tested Phytoestrogens Compared to DES and E2

Ligand	ER α	ER β
DES, E2	Full potent ^b agonist	
ZEA	Mixed potent agonist/antagonist	Mixed Agonist/antagonist
COUM	Full agonist	
GEN	Full, weak agonist, Full agonist	
Eqol	Full, very weak agonist, Full, weak agonist	
RESV	Mixed weak agonist/antagonist	
ENL, 6OH-ENL	Partial agonist/antagonist	
347-IF, 674-IF	Full very weak superagonist	

^aCompilation of (anti)-estrogenic activities are based on data of ligand binding to ER α /ER β and transactivation or inhibition of ER α /ER β presented here.

^bPotent means more than 10% potency compared to DES; agonist means less than 10% but more than 1% potency compared to DES; weak means less than 1% but more than 0.1% potency compared to DES; very weak means less than 0.1% potency compared to DES.

Table 3 summarizes our results on the estrogenic characteristics of the phytoestrogens tested. In the former study, GEN and COUM displayed a preference for ER β (Kuiper *et al.*, 1998). We confirmed that GEN, COUM, and equol have a distinctively higher binding affinity but only slight preference for transactivation of ER β compared to ER α . However, the physiological relevance, if any, for this ER β propensity remains elusive. The potencies of these phytoestrogens were 10- to 100-fold less than that of DES. In contrast, ZEA was the most potent phytoestrogen tested and activated preferentially ER α . All other phytoestrogens tested including RESV and the daidzein and ENL metabolites were weak to very weak ER agonists (Table 3). The daidzein metabolites 347-IF and 467-IF exhibited an interesting and rather surprising feature. Both isoflavones were very weak ER agonists, eliciting no activity up to a dose of 1 μ M, but were superagonists compared to DES at doses of 10 to 100 μ M. The pure antiestrogen ICI completely blocked 347-IF- and 467-IF-induced activity (data not shown), indicating that the measured ER superagonism is due to ligand-dependent activation.

Since the expression of ER target genes depends on the promoter and ERE sequences (reviewed in Klinge, 2001), we analyzed ER activity on a synthetic consensus ERE (3 \times ERE) and the promoter of the endometrial estrogen-responsive C3 gene (Norris *et al.*, 1996). A reporter with multiple copies of ERE, like the 3 \times ERE, enabled the detection of very weakly potent xenoestrogens that may lack measurable activity on natural, single EREs. Nevertheless, we had previously shown that imperfect single EREs might in fact increase estrogen-induced activity of ER α and ER β compared to the consensus ERE (Hall *et al.*, 2002). Whether this observation can be extrapolated to an increased induction of target genes that contain imperfect EREs remains to be analyzed. In the present study, all tested

phytoestrogens showed reduced potencies compared to DES and E2 on the C3 promoter; this is likely due to the lower sensitivity of the full-length C3 promoter compared to the 3 \times ERE reporter (Hall and Korach, 2002). ZEA was the most prominent phytoestrogen with regard to lower potency on the C3 promoter, an estrogen-responsive endometrial marker, for ER β , despite being the most potent phytoestrogen tested. Whether this ERE dependency for ZEA may result in tissue-specific effects has to be evaluated *in vivo*. Taken together, these results strengthened the notion that phytoestrogens are likely to exert tissue-specific effects. However, to provide more conclusive evidence for a potential tissue specificity of phytoestrogens, a broad panel of tissue-specific markers should be investigated (Cassidy, 1999).

Beneficial effects on estrogen-related diseases are often attributed to antiestrogenic activities of phytoestrogens (Bingham *et al.*, 1998). Analysis of antagonistic activity on ER α and ER β revealed that only RESV and ZEA displayed apparent inhibitory properties on both ER α and ER β transactivation. The partial agonists ENL and 6OH-ENL were also partial antagonists in accordance with basic pharmacological principles. Bowers and colleagues reported that RESV showed slight antagonistic properties on ER α but not ER β depending on the ERE sequence (Bowers *et al.*, 2000). We observed an increase of DES-induced activity on the consensus ERE by both ER α and ER β at doses up to 10 μ M for RESV. At 100 μ M, RESV repressed activity of both ER α and ER β . The apparently contrasting results observed with ER β are likely due to the lower maximum dose of 50 μ M tested in the former study and/or the use of different ERE sequences (Bowers *et al.*, 2000). The ER α antagonistic properties of RESV were confirmed in mammary tumor models as well as in Ishikawa cells with endogenous ER α expression (Bhat *et al.*, 2001; Bhat and Pezzuto, 2001). Taken together, the published reports and our data support that RESV has the potential to act as a mixed agonist/antagonist in an ERE- and dose-dependent fashion, and this may, next to its nonestrogenic effects (Dong, 2003), account for RESV's beneficial effects. ZEA was a more potent antiestrogen than RESV and lacked any additive agonistic effects with DES on ER α and ER β , indicating its potential to act as a pure ER antagonist at high doses.

Recruitment of coactivators to ER α and ER β is a prerequisite for ER action, and ER coactivators are also important determinants for tissue-specific estrogen action, since coregulators show a tissue-specific expression profile (reviewed in Tremblay and Giguere, 2002). ER ligands regulate recruitment of coactivators by inducing a distinct conformation of the ligand binding and activation domains of the ERs (Paige *et al.*, 1999). Thus, it is reasonable to assume that a conformational change induced by different phytoestrogens caused the observed distinct transactivation of ER α and ER β (Hall *et al.*, 2002). We showed that all ER agonists tested enhanced recruitment of coactivators at doses similar to that which induced transactivation. This confirmed that coactivator recruitment is necessary for ER action and that a

two-hybrid coactivator assay could serve as surrogate marker for ER activation (Nishikawa *et al.*, 1999).

Previous studies analyzed coactivator recruitment to ER α and ER β in the presence of phytoestrogens using glutathione-S-transferase pull-down or yeast two-hybrid assays (An *et al.*, 2001; Nishikawa *et al.*, 1999; Routledge *et al.*, 2000). These studies showed that ER β -selective phytoestrogens like genistein were more potent to recruit coactivators to ER β compared to ER α . We also observed maximum recruitment of coactivators to ER β at lower doses of GEN and COUM, albeit with lower efficacy, compared to ER α . All the rather potent phytoestrogens we studied enhanced preferentially recruitment of GRIP1 to ER α , confirming the report by Nishikawa and colleagues (1999). This phytoestrogen-induced GRIP1 propensity of ER α was similar to that induced by E2, indicating that the molecular mechanism of ER α agonist activity of the phytoestrogens tested is comparable to potent estrogens. In contrast, the weakly potent superagonists 347-IF and 467-IF did not preferentially recruit one coactivator. For ER β , no distinct preference for one coactivator was apparent and the overall association was less pronounced than for ER α . This confirmed the generally observed lower efficacy of ER β compared to ER α regardless of the higher ER β affinity and potency of some phytoestrogens tested. The ER antagonists ZEA and RESV repressed GRIP1 association to ER α to a greater extent than for SRC-1, confirming their agonist profiles. Interestingly, RESV displayed the typical inverted U-shaped dose response for ER β antagonism for SRC-1 association but not for GRIP1. Also, GRIP1 association with ER β was diminished at low, agonistic doses of RESV. Taken together, these findings indicate that SRC-1 rather than GRIP1 may contribute to RESV's weak agonistic activity on ER β at lower doses.

The reported agonistic and antagonistic activity of the phytoestrogens we studied can aid in the evaluation of potential hazards or benefits of these compounds. Enterolactones are highly abundant in flaxseeds and lentils, food more common in a western diet, while isoflavones found in soy and tofu are generally considered part of an eastern diet (Cassidy *et al.*, 2000). Due to their abundance (Cassidy *et al.*, 2000; Munro *et al.*, 2003) and potencies, the soy-derived GEN, COUM, the daidzein metabolites equol and the superagonists 347-IF and 467-IF, RESV found mainly in grapes, and the mycotoxin ZEA seem to be relevant as far as potential risk/benefit of their (anti)-estrogenic effects. The isoflavones showed all pure ER agonistic activity. Thus, these compounds should be regarded as potentially estrogenic and, consequently, as potential endocrine disruptors that may cause elevated cell proliferation leading to estrogen-dependent tumor promotion (Allred *et al.*, 2001; Cotroneo *et al.*, 2002; Hilakivi-Clarke *et al.*, 1999a; Newbold *et al.*, 2001) and that may also induce adverse developmental effects (Delclos *et al.*, 2001). The beneficial effects associated with soy intake are likely due in part to non-ER-mediated effects as described above. But, with regard to the adverse and beneficial ER-mediated effects, the timing of

exposure is important (Bouker and Hilakivi-Clarke, 2000; Cotroneo *et al.*, 2002). In contrast, RESV's very weak agonistic properties together with its antagonistic properties are likely to be major contributors to the beneficial effects attributed to RESV (Bhat *et al.*, 2001). ZEA might act as an endocrine disruptor at low doses, but its ER antagonistic activity at high doses might contribute to the observed reduction of mammary tumors (Hilakivi-Clarke *et al.*, 1999b). Next to the direct effects on ER activity that were analyzed in this study, phytoestrogens might also affect the formation of endogenous estrogens like 17 β -estradiol. One well-characterized and potential target that regulates estradiol formation is aromatase, the enzyme that catalyzes the conversion of testosterone to estradiol. Indeed, indirect evidence for antiestrogenic effects due to inhibition of the formation of 17 β -estradiol has been provided for biochanin A but not genistein at low doses (Almstrup *et al.*, 2002).

In conclusion, the risks and benefits of estrogenic or antiestrogenic effects depend highly on the target tissue as well as the timing and level of exposure. These latter two factors along with further research on the potential tissue-specific effects of phytoestrogens should aid in the assessment of the real risks and benefits of phytoestrogen-containing diets.

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