Interactions of Dietary Estrogens with Human Estrogen Receptors and the Effect on Estrogen Receptor–Estrogen Response Element Complex Formation

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Epidemiologic and experimental studies support the hypothesis that dietary estrogens from plant sources (phytoestrogens) may play a role in the prevention of breast and prostate cancer. The molecular mechanisms for such chemopreventive effect are still unclear. We investigated the possibility that phytoestrogens may bind differentially to estrogen receptor proteins (ERα and ERβ) and affect the interactions of the ligand–ER complexes with different estrogen response element (ERE) sequences. We used fluorescence polarization to measure the binding affinities of genistein, coumestrol, daidzein, glyceollin, and zearalenone for human ERα and ERβ. Competition binding experiments revealed higher affinity of the phytoestrogens for ERβ than for ERα. Genistein [median inhibitory concentration 12nM] is the most potent and has the same relative binding affinity for ERβ as 17β-estradiol. We also studied the effect of these phytoestrogens on the ability of ERα and ERβ to associate with specific DNA sequences (EREs). The direct binding of human recombinant estrogen receptors to fluorescein-labeled EREs indicates that phytoestrogens can cause conformational changes in both human ERs, which results in altered affinities of the complexes for the ERE from the Xenopus vitellogenin A2 gene and an ERE from the human *pS2* gene. *Key words*: cancer chemoprevention, dietary estrogens, estrogen receptor, estrogen response element, fluorescence polarization, phytoestrogens, xenoestrogens. *Environ Health Perspect* 108:867–872 (2000). [Online 1 August 2000]
http://ehpnet1.niehs.nih.gov/docs/2000/108p867-872nikov/abstract.html

Estrogen is a steroid hormone, which influences the growth, differentiation, and function of many target tissues. These include tissues of the female and male reproductive systems such as mammary gland, uterus, vagina, ovary, testes, and prostate. Estrogens play an important role in bone maintenance, in the central nervous system, and in the cardiovascular system (*1*). Estrogens are also involved in the development of breast and endometrial cancers; in addition, they may have important roles with regard to prostate and colon cancers (*2*). The effects of estrogen are mediated by two receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ). Both receptors are members of the superfamily of nuclear receptors and have high degrees of homology in their ligand-binding domains (LBDs) and DNA-binding domains (DBDs) (*3,4*). ERα and ERβ have similar affinities for 17β-estradiol (E_2), recognize a consensus DNA estrogen response element (ERE) located within the regulatory region of target genes (*4,5*), and are expressed in distinct and overlapping tissues (*6*) as well as during human tumorigenesis (*7*). In the absence of hormone, the ER resides in the nucleus of target cells where it is associated with the heat-shock proteins hsp90 and hsp59 (*8,9*). The binding of E_2 to ER is followed by a conformational change, leading to dissociation of the receptor from the heat-shock proteins, formation of stable receptor dimers (*10*), and subsequent interaction with the ERE. The DNA-bound receptor can then either positively or negatively regulate target gene expression (*11*). Although the precise mechanism by which the ER modulates RNA polymerase activity remains to be determined, the agonist-bound ER can recruit accessory proteins that permit the receptor to activate the transcriptional apparatus (*11–13*). Conversely, when occupied by antagonists, the ER either does not bind ERE or the DNA-bound receptor associates with corepressor proteins that repress transcription (*12*).

The human diet contains several nonsteroidal estrogenic compounds, which are structurally similar to natural and synthetic estrogens and antiestrogens. Dietary estrogens are either produced by plants themselves (phytoestrogens) or by fungi that infect plants (mycoestrogens). Phytoestrogens can be divided into three main classes: isoflavones (such as genistein and daidzein), coumestans (such as coumestrol), and lignans (such as enterodiol and enterolactone) (Figure 1). Soybeans and clover, as well as other legumes, are the most significant sources of isoflavones and coumestans (*14*). In response to pathogens and other stimuli, soybean tissues accumulate the phytoalexin glyceollin, which shares structural similarities with the isoflavones (*15*). Mycoestogens include primarily zearalenone (resorcylic acid lactone) and its derivatives (*14*). Dietary intake of phytoestrogens is significantly higher in countries where the incidence of breast and prostate cancers is low, suggesting that they may act as chemopreventive agents (*16*). The chemopreventive effect of dietary soy has been demonstrated on the development of induced mammary tumors in rodents (*16*). Phytoestrogens are believed to exert their chemopreventive action by interacting with the ERs and thus modulate the transcription of target genes, although alternative mechanisms have also been proposed (*14*).

In this study we used fluorescence polarization (FP) to investigate the estrogenic activity of isoflavones, coumestans, phytoalexins, and mycoestrogens in competition binding assays with human ERα and ERβ. We also investigated the ability of the liganded receptors to interact with Xenopus vitellogenin (*vit)* A2 ERE and human *pS2* ERE in direct binding assay.

FP is used to study molecular interactions by detecting the changes in the effective molecular volume of fluorescent molecules (*17,18*). When plane-polarized light is used to excite a solution of fluorescent molecules, the molecules parallel to the plane become excited. The molecules in solution tumble during the period of excitation and thus the emitted light is depolarized. The observed polarization is a measure of the tumbling rate of the fluorescent molecule and is directly related to its molecular volume (*17–19*). Changes in the molecular volume that result from binding, dissociation, or conformational changes are detected by FP. If a fluorescent molecule becomes bound to another molecule, the larger complex will tumble slower than the free fluorescent molecule and high polarization values will be measured. There are several methods for measuring ligand–receptor binding interactions (*20,21*), but we chose FP because it can be run at room temperature, requires only hours to complete, involves no radioactivity, and can be used for screening of weak estrogens with limited solubility (*17,18*).

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This research was supported by grant USDA 58-6435-7-019 to the Tulane/Xavier Center for Bioenvironmental Research from the U.S. Department of Agriculture and grant LEQSF (1997-00)-RD-B-12 from the Louisiana Board of Regents to W.L.A.

Received 17 February 2000; accepted 3 May 2000.

Materials and Methods

Materials. The steroids E_2 and testosterone were obtained from Sigma Chemical Co. (St. Louis, MO).

The phytoestrogens genistein (4´,5,7-trihydroxyisoflavone), daidzein (4´,7-hydroxyisoflavone), coumestrol [2-(2-,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid δ-lactone], and zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone] were purchased from Indofine Chemical Company, Inc. (Belle Mead, NJ). Glyceollin was obtained from the U.S. Department of Agriculture Southern Regional Research Center.

Human recombinant estrogen receptors α and β, and fluorescein-labeled E_2 (ES2) were purchased from Pan Vera Corporation (Madison, WI). Fluorescein end-labeled Xenopus *vit* A2, and human *pS2* EREs and glucocorticoid response elements (GRE) were custom synthesized by Oligos Etc. (Wilsonville, OR).

ES2–ER direct binding experiments. Recombinant human ERα and ERβ (PanVera Corporation) were serially diluted from 256 nM to 0.5 nM in screening buffer (100 mM potassium phosphate, pH 7.5; 100 µg/mL bovine gamma globulin; 0.02% sodium azide) to a final volume of 100 µL in borosilicate test tubes. ES2 (fluorescein-labeled E_2) was added to each test tube to a final concentration of 1 nM and incubated for 60 min at room temperature. The FP of each tube was measured on a Beacon 2000 Fluorescence Polarization Instrument (PanVera Corporation) with 490 nm excitation filter and 530 nm emission filter (*18,19*). FP values were plotted versus ER concentration.

We used a nonlinear least-squares curve fitting program (Prizm; Graphpad Inc., San Diego, CA) to calculate the dissociation constant (K_d) as the concentration of ER at which half of the ligand is bound.

Competitive binding experiments. We tested genistein, daidzein, coumestrol, glyceollin, and zearalenone to determine their ability to displace the ES2 molecule from ERα–ES2 and ERβ–ES2 complexes.

We prepared serial dilutions of each competing phytoestrogen from an 8 mM ethanol stock solution in screening buffer. Preincubated ERα or ERβ (13 nM) and ES2 (1 nM) were added to produce a final volume of 100 µL. After 60 min incubation at room temperature, the polarization values at each competitor's concentration were measured using the Beacon 2000 FP system with 490 nm excitation filter and 530 nm emission filter. The polarization values were converted to percent inhibition using the equation

$$I_{\%} = (P_0 – P)/(P_0 – P_{100}) \times 100,$$

where P_0 is the polarization value at 0% inhibition, P_{100} is the polarization value at 100% inhibition, and P is the observed FP at each concentration point. We used free ES2 (100% inhibition) as a positive control and ER–ES2 complex (0% inhibition) as a negative control. We transformed polarization values into percent inhibition to normalize the differences at 0% inhibition for each run. We then analyzed the percent inhibition versus competitor concentration curves by nonlinear least-squares curve fitting and determined the concentration of competitor needed to displace half of the bound ligand (IC_{50}). To compare the binding affinities of the tested phytoestrogens, we converted IC_{50} values to relative binding affinities (RBA) using E_2 as a standard. The E_2 RBA was set equal to 100, and the RBA value for each of the phytoestrogens was calculated using the following formula:

$$\text{RBA} = (IC_{50}\ E_2/IC_{50}\ \text{competitor}) \times 100.$$

ERE preparation. We tested ERE from the Xenopus *vit* A2 gene, ERE from the human *pS2* gene, and consensus GRE to bind ERα and ERβ (Table 1). The sense DNA strands (Oligos Etc., Wilsonville, OR) containing EREs and GRE were labeled with fluorescein attached via a 6-carbon spacer at the 5´ terminus (*22*). The 35-base pair double stranded oligonucleotides were prepared by annealing equimolar concentrations of the sense and antisense strands in 10 mM Tris-HCl, pH 7.8, and 150 mM NaCl. This mixture was heated to 95°C for 10 min and slowly cooled (30 min) to room temperature. To remove any hairpin formations, we purified the double stranded DNA by using 12% polyacrylamide (1:19 bisacrylamide:acrylamide) gel electrophoresis containing 89 mM Tris-borate; 2.5 mM EDTA, pH 8.3; and 10% ammonium persulphate (*23,24*).

ER–ERE direct binding studies. To further investigate the estrogenic properties of the phytoestrogens, we performed direct binding experiments and measured the abilities of ERα and ERβ to associate with Xenopus *vit* A2 ERE and human *pS2* ERE in the presence of phytoestrogens. ERα and ERβ were serially diluted from 450 nM to 0.8

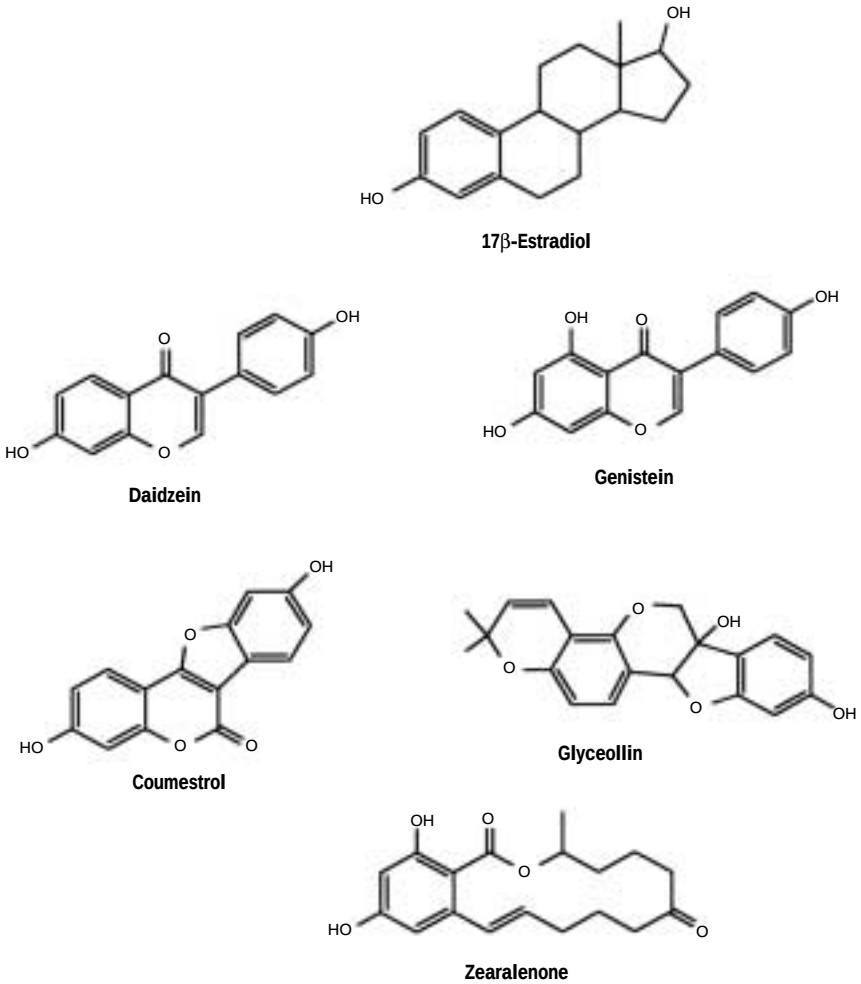

Figure 1. Structures of 17β-estradiol and all dietary estrogens used in this study.

nM in DNA binding buffer (10 mM potassium phosphate, pH 7.8; 0.1 mM EDTA; 50 µM magnesium chloride; 10% glycerol). We incubated the ERs 30 min with concentrations of each of the phytoestrogens required to saturate ERα and ERβ as determined by competitive binding experiments, and then for 10 min with poly (dI-dC) (1 µg/5 µg of protein) at room temperature. The binding, initiated by adding fluorescein-labeled synthetic oligonucleotide EREs (final concentration 0.5 nM), was allowed to proceed at room temperature 60 min. The polarization values of each ER concentration were then measured on a Beacon 2000 instrument with 490 nm excitation and 530 nm emission maximums. We constructed the binding isotherm by plotting percent saturation versus ER concentration using the formula

$$S_{\%} = (P–P_0)/(P_{100}–P_0) \times 100,$$

where P_0 is the polarization value at 0% saturation, P_{100} is the polarization value at 100% saturation, and P is the observed FP at each concentration point. We calculated the K_d from the binding curves using a nonlinear least-squares curve fitting program. The binding affinities of ERα and ERβ (liganded with phytoestrogens) for EREs were also calculated in terms of RBA [RBA = (K_d E_2/K_d competitor) × 100].

To prove the reliability and specificity of the method, we compared the binding affinities of ERα and ERβ (liganded with E_2) for fluorescein-labeled Xenopus *vit* A2 ERE and fluorescein-labeled GRE (Figure 2). At the concentration range tested, no ER–GRE complexes were formed, as opposed to the high affinity binding of both ERs to the consensus ERE.

Results

ERα and ERβ saturation with ES2. Figure 3 shows the curves of ES2 saturation binding to recombinant human ERs. We titrated 1 nM labeled ligand with increasing concentrations of the ERs to produce these binding isotherms. The K_d values calculated from the saturation curves were 10 nM for ERβ and 25 nM for ERα. The affinity of labeled ES2 ligand was 2-fold higher for ERβ than for ERα.

Binding affinities of several phytoestrogens for ERα and ERβ. We determined the binding affinities of different classes of phytoestrogens for ERα and ERβ in competition binding with the ER–ES2 complex. We determined the binding affinities (IC_{50} values) of the tested dietary estrogens from the competition curves (Figure 4 and Table 2). Phytoestrogens compete with ES2 for binding ERα in the following order: zearalenone > coumestrol > genistein > glyceollin > daidzein; for ERβ: genistein > zearalenone > coumestrol > daidzein > glyceollin (Table 2).

With the exception of glyceollin, the affinity of all the dietary estrogens tested is much higher for ERβ than ERα. The binding affinity of genistein for ERβ is 60-fold higher than its affinity for ERα, whereas for coumestrol and zearalenone, there is approximately a 3-fold difference (Table 2). Glyceollin was found to have a 3-fold higher affinity for ERα. E_2 binds to ERα with an affinity approximately 3-fold higher than genistein, which has been previously observed (*25*). Genistein binds with the same affinity as E_2 to ERβ. Zearalenone shows similar activity as genistein and forms a complex with ERβ with 1.3-fold less affinity than E_2.

Phytoestrogen-dependent binding of ERα and ERβ to two different EREs. All of the phytoestrogens promote less binding of ERα and ERβ to Xenopus *vit* A2 ERE than does E_2(Table 3). Genistein and zearalenone cause similar changes in the affinity of both receptors for this consensus ERE, which is approximately 2-fold lower than the effect of E_2. We observed approximately 2-fold higher affinity for the binding of the coumestrol–ERα complex to Xenopus *vit* A2 ERE than for the binding of the coumestrol–ERβ complex. This is also true for ER complexes with daidzein, but daidzein–ERβ complex has almost 6-fold higher affinity than daidzein–ERα complex. Glyceollin inhibits the formation of ER–ERE complexes (Table 3).

The binding of ERα to human *pS2* ERE in the presence of E_2 occurs with approximately 2.5-fold higher affinity than the binding of ERβ in the presence of E_2, (K_ds of 32 nM and 84 nM, respectively). Zearalenone produces high affinity binding of both ERs to human *pS2* ERE, with zearalenone–ERβ complex binding even more tightly than E_2–ERβ complex. Genistein is the only phytoestrogen we tested that causes a considerable difference in the binding of the ERα and ERβ complexes to the human ERE. The ERβ complex containing genistein has approximately 2-fold lower affinity than the genistein–ERα complex.

We observed similar affinity of ERα for both EREs when ERα was liganded with E_2, but we found differential binding of ERα to the EREs in the presence of the dietary estrogens. Zearalenone, genistein, and daidzein cause better binding of ERα to human *pS2* ERE as compared with Xenopus *vit* A2 ERE. Coumestrol is the only phytoestrogen we tested that induces a higher affinity binding of ERα to the Xenopus *vit* A2 ERE (Table 3).

E_2 promotes differential binding of ERβ to the EREs, with 2.5-fold higher affinity of the E_2–ERβ complex for Xenopus *vit* A2 ERE. The relative binding affinities of ERβ for both EREs are similar when the receptor is liganded with coumestrol, genistein, and daidzein. Zearalenone–ERβ complex differentially binds to the EREs; the complex has higher relative binding affinity for human *pS2* ERE than E_2–ERβ.

The K_ds and relative binding affinities alone do not fully describe the interactions

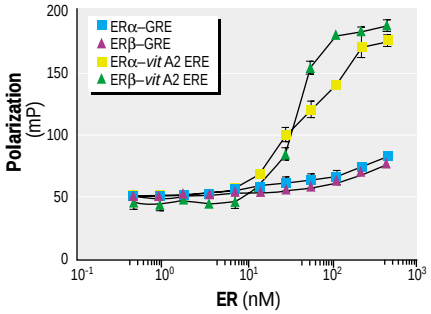

Figure 2. Binding of ER–E_2 complexes to GRE and Xenopus *vit* A2 ERE. mp, millipolarization. Data points represent the mean percent saturation value ± SEM from two different experiments.

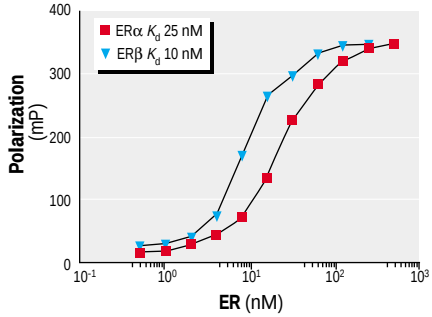

Figure 3. The binding isotherms of human recombinant ERα and ERβ and ES2. mp, millipolarization. The fluorescence of ES2 alone produces low polarization values. Upon binding ER molecules, the rotational freedom of the complex decreases and higher polarization values are measured. When all ES2 molecules are bound, further increase in the ER concentration does not affect the FP of the system. Data points represent the mean polarization value ± SEM from two different experiments.

Table 1. Double stranded DNA sequences containing EREs and GRE.

Xenopus *vit* A2 ERE	5′ XGTC CAA AGT CA GGTCA CAG TGACC TGA TCA AAG TT 3′ 3′ CAG GTT TCA GT CCAGT GTC ACTGG ACT AGT TTC AA 5′
Human *pS2* ERE	5′ XGT CCA AAG TCA GGTCA CGG TGGCC TG ATC AAA GTT 3′ 3′ CA GGT TTC AGT CCAGT GCC ACCGG AC TAG TTT CAA 5′
Consensus GRE	5′ XGT CCA AAG TCA GAACA CAG TGTTC TGATC AAA GTT 3′ 3′ CA GGT TTC AGT CTTGT GTC ACAAG ACTAG TTT CAA 5′

bp, base pair. The underlined sequences represent the limits of the 13 bp reverse repeat of the EREs and the GRE that contain 5 bp arms and a 3 bp spacer region.

of ERα and ERβ with the EREs, especially at end point saturation concentrations (Figure 5). The maximal effective molecular volume of Xenopus *vit* A2 and human *pS2* EREs varies with the ER–phytoestrogen complex present. The difference is more profound with ERβ than with ERα (Figure 6). Coumestrol and genistein trigger similar changes in ERβ, which result in different effective molecular volumes of the labeled response elements. ERβ liganded with either of the two phytoestrogens triggers 50% faster rotational motion (decreased molecular volume) of Xenopus *vit* A2 ERE than the rotational motion of this ERE in the presence of the ERβ–E_2 complex. The speed of rotation of human *pS2* ERE in the presence of ERβ–genistein complex or ERβ–coumestrol complex is decreased to approximately 50% (increased molecular volume) from its rotational motion with ERβ–E_2 complex present (Figure 6). We also observed the same pattern with ERα, but the differences at end point saturation concentrations are not as significant as with ERβ.

Discussion

We used the FP method to study the interactions of several phytoestrogens with human ERα and ERβ and their effects on ER–ERE complex formation. This approach allows detection of ligand–receptor and receptor–response element interactions in solution (without solid supports) and at room temperature. The information obtained can be analyzed by nonlinear least-squares curve fitting to yield the binding constants of these interactions.

In several epidemiologic studies, a relationship between the intake of soy foods and reduced breast or prostate cancer has been suggested (*26–30*), and one of the proposed mechanisms involves activation of transcription through the ERs. In our studies, we used recombinant human ERs and labeled E_2 to compare the affinities of different classes of phytoestrogens to bind ERα and ERβ. The isoflavone genistein, the coumestan coumestrol, and the resorcylic acid lactone zearalenone have greater affinity for both receptors than daidzein and glyceollin (Figure 4).

This can be explained by the size of the binding cavity of the ER, which has a volume almost twice that of the E_2 molecule. The length and the width of the E_2 skeleton are very well matched by the receptor's ligand binding domain, but there are large unoccupied spaces opposite the B-ring and the C-ring of E_2 (*31*). Previous studies found that coumestrol has the highest affinity for both receptors (*25*), but this was not confirmed by our competitive binding experiments. These FP competition binding experiments were performed at room temperature and the phytoestrogens were incubated with the ER–ES2 complex for 2 hr, whereas in the competition binding method described by Kuiper et al. (*25*), the phytoestrogens were incubated with ^{3}H-E_2–ER complex for 18–20 hr at 6°C. These differences in binding times and temperatures, and the fact that we used an E2S with an increased molecular volume instead of ^{3}H-E_2, may account for the different relative binding affinities that we observed. The FP measurements also indicate that genistein has greater binding affinity for ERβ than does coumestrol, whereas zearalenone has greater binding affinity for ERα (Table 2).

We observed differential binding of the dietary estrogens to the receptor proteins. FP indicates that genistein binds ERβ with the same affinity as E_2 and has low relative binding affinity for ERα. Differences in the binding to both human receptors were also observed with zearalenone and coumestrol. This differential binding may suggest tissue-specific biologic effects triggered by the dietary estrogens because both ER subtype transcripts were found in breast and prostate tumor tissues, but with different expression levels (*32*,*33*).

To better understand the influence of the dietary estrogens on ER–ERE complex formation, we compared the relative binding affinities of the receptor proteins liganded with phytoestrogens for the consensus ERE derived from Xenopus *vit* A2 gene and a human *pS2* ERE (Table 1). ERα saturated with any of the phytoestrogens has lower affinity for both EREs than ERα liganded with E_2. Coumestrol promotes the highest affinity of ERα for Xenopus *vit* A2 ERE, approximately 1.5-fold less than the effect triggered by E_2. The same phytoestrogens differentially affect the binding of ERα to human *pS2* ERE, and zearalenone influences binding with a magnitude almost as potent as E_2 (Table 3). We found that phytoestrogens

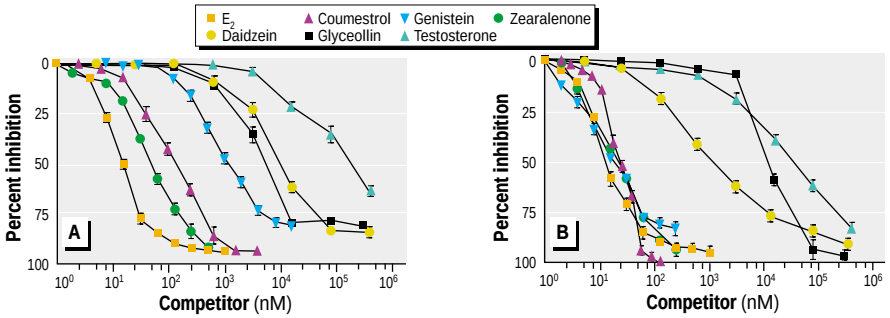

Figure 4. Competition binding curves of various dietary estrogens for (*A*) ERα–ES2 and (*B*) ERβ–ES2 complexes. The initial ER–ES2 complexes have high polarization values. When the complex is titrated with competitors, ES2 molecules are displaced from the ER and there is a gradual decrease in the polarization values. Data points represent the mean percent inhibition value ± SEM from two different experiments.

Table 2. RBAs and IC_{50} constants of tested dietary estrogens for human ERα and ERβ from competition experiments.

	ERα		ERβ	
Compound	IC_{50}	RBA	IC_{50}	RBA
E_2	13 ± 0.7 nM	100	12 ± 0.5 nM	100
Genistein	825 ± 2 nM	1.6	12 ± 0.7 nM	100
Coumestrol	109 ± 1 nM	12	35 ± 0.7 nM	34
Zearalenone	59 ± 0.8 nM	22	16 ± 0.5 nM	75
Daidzein	7 ± 1 μM	0.2	670 ± 1 nM	1.8
Glyceollin	6 ± 0.6 μM	0.22	16 ± 1.4 μM	0.08
Testosterone	35 ± 0.5 μM	0.04	20 ± 1 μM	0.06

The RBA of each competitor was calculated as a ratio of the IC_{50} values of each competitor and E_2. The RBA value of E_2 was arbitrarily set at 100. The data represent the mean IC_{50} values ± SEM from two different experiments.

Table 3. K_d constants and (RBAs) of ERα and ERβ (saturated with phytoestrogens) for Xenopus *vit* A2 and human *pS2* EREs.

	Xenopus *vit* A2 ERE		Human *pS2* ERE	
Compound	ERα	ERβ	ERα	ERβ
E_2	32 nM (100)	34 nM (100)	32 nM (100)	84 nM (100)
Genistein	57 nM (56)	70 nM (49)	42 nM (76)	212 nM (40)
Coumestrol	45 nM (71)	97 nM (35)	75 nM (43)	218 nM (39)
Zearalenone	57 nM (56)	69 nM (49)	34 nM (94)	70 nM (120)
Daidzein	209 nM (15)	40 nM (85)	50 nM (67)	118 nM (71)
Glyceollin	– (< 0.01)	– (< 0.01)	– (< 0.01)	– (< 0.01)

The RBA of each ER saturated with competitor was calculated as a ratio of the K_d values of each competitor and E_2. The RBA values of the ERs saturated with E_2 were arbitrarily set at 100.

have similar effects on the relative binding affinities of ERβ to the *vit* A2 and *pS2* response elements, and only zearalenone causes differences in the binding of the receptor to the EREs. The data suggest that, upon binding those structurally different phytoestrogens, the receptor proteins undergo conformational changes, which differentially affect the formation of the ER–ERE complexes. The dietary estrogens apparently induce distinct conformational changes in ERα and ERβ, as have been previously observed with other ER ligands (*34*).

Apart from the relative binding affinities of the ERs for the response elements, the changes in the effective molecular volume of the Xenopus *vit* A2 and the human *pS2* response elements triggered by different ERβ–phytoestrogen complexes (Figure 6) provide additional information about the interactions of the receptor proteins with DNA. The molecular volume of Xenopus *vit* A2 ERE complexed with ERβ–genistein or with ERβ–coumestrol is only about half the effective molecular volume of the complex of this ERE with ERβ–E_2. The molecular volume of human *pS2* ERE is also affected by ERβ–genistein and ERβ–coumestrol complexes; it is approximately 1.5-fold higher than the molecular volume of human *pS2* ERE complexed with ERβ–E_2 (Figure 6). We do not yet know the exact reason for the high polarization values (decreased speed of rotation of the labeled molecule) due to the binding of ERβ–genistein/coumestrol to human *pS2* ERE at high protein concentrations. Because the FP depends on the rotational freedom of the fluorescent molecule, especially the fluorescence label (*17*,*19*), it is possible that binding of the receptor protein–phytoestrogen complex changes the geometry of the labeled DNA molecule, for example, by increasing the bending of the DNA chain or by causing a partial unwinding (loosening) of the end of the DNA that is labeled with fluorescein. The data, however, clearly demonstrate that phytoestrogens affect differentially the ER–ERE interactions. Based on these findings we conclude that phytoestrogens interact with the human ERs in a manner that influences both the formation and the physical properties of ER–ERE complexes. We were able to detect these differences in ER–ERE complex formation using EREs from Xenopus *vit* A2 and human *pS2* genes that differ with only one base pair (Table 1). Functional and nonfunctional EREs with one or two base pair differences can be found in many genes (*35*), and differences in the conformation of the ERs complexes with xenoestrogens may cause transformations of different functional EREs into nonfunctional ones and vice versa.

Kinetics of the frequency of ER–DNA interactions in the presence of different ER ligands have been studied by Cheskis et al. (*36*). They found that ligand binding affects the kinetics of human ERα interaction with Xenopus *vit* A2 ERE. They also found that E_2 induces rapid formation of an unstable ER–ERE complex, whereas binding of

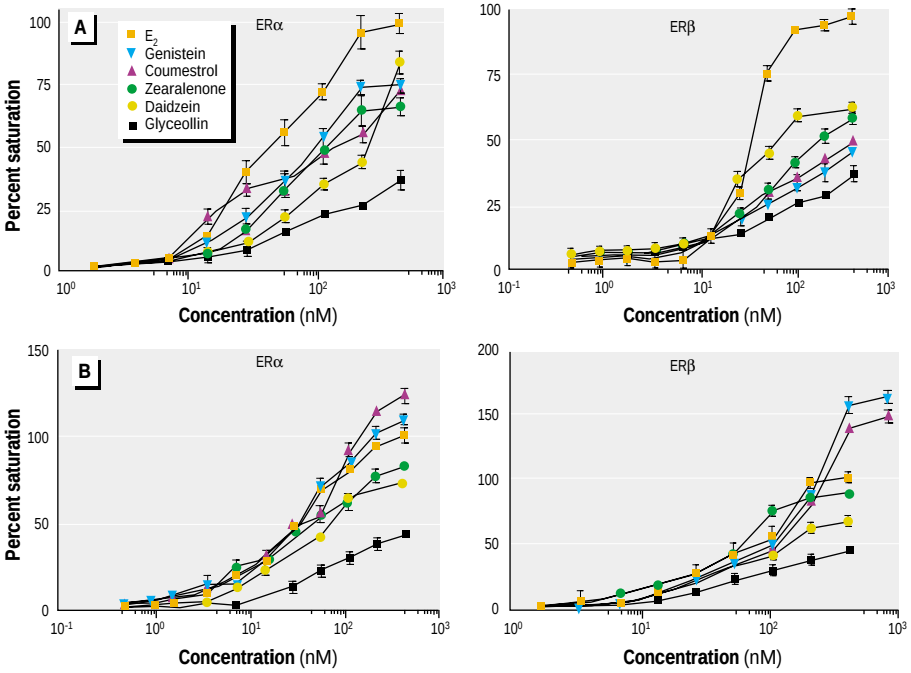

Figure 5. Binding of human recombinant ERα and ERβ (saturated with various phytoestrogens) to (*A*) fluorescein-labeled Xenopus *vit* A2 ERE, and (*B*) fluorescein-labeled human *pS2* ERE. Data points represent the mean percent saturation value ± SEM from two different experiments.

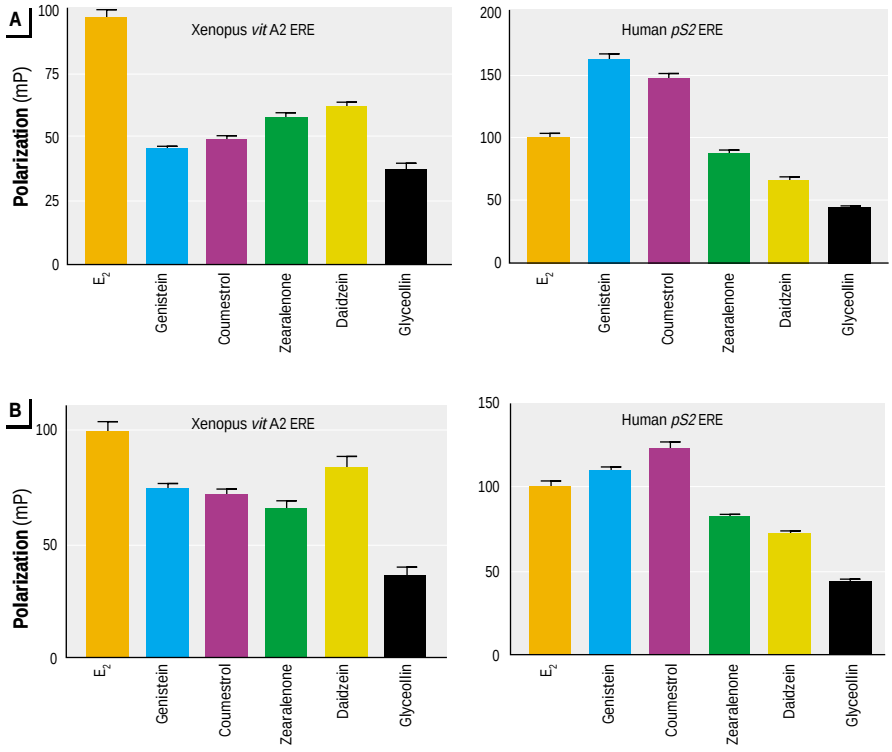

Figure 6. Speed of rotation (represented as polarization values) of the labeled consensus and nonconsensus EREs in the presence of (*A*) human recombinant ERβ and (*B*) human recombinant ERα, both liganded with E_2 and various phytoestrogens. mp, millipolarization. The speed of rotation of each of the EREs complexed with ERβ–E_2 or ERα–E_2 was arbitrary set at 100. The data represents the mean FP value ± SEM from two different experiments.

"pure" antagonist such as ICI 182,780 results in a slow formation of a very stable receptor–DNA complex. Cheskis et al. (*36*) concluded that the kinetics of ligand binding to EREs were correlated with the observed biologic activities of the ligands. Our data also support the view that ligand binding may induce conformational changes that not only modulate the interactions of ER with other transcriptional factors but directly affect the physical properties of ER–ERE complexes.

References and Notes

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