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Phytoestrogens Modulate Binding Response of Estrogen Receptors α and β to the Estrogen Response Element

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Binding of estrogen receptor (ER) to estrogen response element (ERE) induces gene activation and is an important step in estrogen-induced biological effects. Here, we investigated the effects of some dietary phytoestrogens such as the isoflavones genistein and daidzein, its metabolite equol, and the coumestane coumestrol on the binding rate of ERα and ERβ to ERE by a nonradioactive real-time method, the Biacore Technology. ERα and ERβ were able to bind to ERE immobilized on the surface of a sensor chip even in the absence of estrogens. 17β-Estradiol and phytoestrogens induced an increase in ER binding to ERE in a concentration-dependent manner. 17β-Estradiol was a more potent activator of binding than the phytoestrogens studied. The concentrations of 17β-estradiol inducing an increase in the binding response of ERα and ERβ to ERE by 50% (EC(50)) as compared to unliganded ER were 0.03 and 0.01 μM, respectively. Regarding the efficacy of activation of ERα, from the most to the least effective compound, the sequence and the EC(50) were as follows: 17β-estradiol (0.03 μM) > coumestrol (0.2 μM) > equol (3.5 μM) > genistein (15 μM) > daidzein (>300 μM) and for ERβ 17β-estradiol (0.01 μM) > coumestrol (0.025 μM) > genistein (0.03 μM) > daidzein (0.35 μM) > equol (0.4 μM). The ratios EC(50)α/EC(50)β were calculated to be for 17β-estradiol, 3; coumestrol, 8; equol, 8.8; genistein, 500; daidzein > 850. These ratios indicate that genistein and daidzein preferentially activate the binding of ERβ to ERE. The endogenous hormone 17β-estradiol as well as coumestrol and daidzein metabolite equol activate the binding of ERβ to ERE only slightly more effectively than the binding of ERα to ERE. Thus, the effect of daidzein can be changed from a specific activator of ERβ to an activator of both ER isotypes α and β in humans who are able to convert daidzein to equol. While the results of the measurements with ERα were in line with the binding affinities of compounds tested for ER, there was a distinct difference between our results and the binding affinities of phytoestrogens for the ERβ. This leads to the conclusion that phytoestrogens differ not only in their binding affinities for the ER, but also in their potential to increase the rate of receptor binding to the ERE.

KEYWORDS: Estrogen receptor; phytoestrogens; estrogen response element; biacore; surface plasmon resonance

INTRODUCTION

Diets rich in phytoestrogens (plant estrogens) are associated with a lower risk of breast and prostate cancer and could play a role in the prevention of some other estrogen-related diseases such as osteoporosis and cardiovascular diseases (*1*). The molecular mechanisms of phytoestrogen action in humans still remain to be investigated. It has been shown that phytoestrogens are able to bind to ERα and ERβ (*2*). However, simple binding cannot predict the phytoestrogen-induced interaction of ER with ERE and activation of gene transcription. In this study we investigated the step that follows the binding of estrogens and phytoestrogens to the receptor, i.e., binding of the activated receptor to the ERE. The aim of the present study was to test whether there are differences between the efficacy of known binding affinities of the phytoestrogens for the ER and the binding rate of 'activated' ERα and ERβ to ERE induced by 17β-estradiol and a number of phytoestrogens. We focused on dietary phytoestrogens genistein and daidzein, their metabolite equol, and a member of the coumestanes coumestrol (**Figure 1**).

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MATERIALS AND METHODS

Reagents and Equipment. The Biacore X system, CM5 sensor chip, HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20, pH 7.4), and the amine coupling kit were obtained

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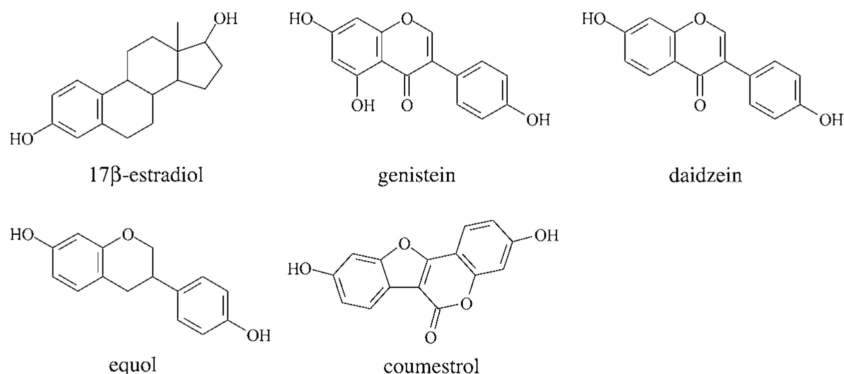

Figure 1. Overview of the tested substances.

from Biacore (Freiburg, Germany). Streptavidin (SA) and 17β-estradiol (E2) were provided by Calbiochem (Darmstadt, Germany), and the biotinylated oligonucleotides were from Carl Roth (Karlsruhe, Germany). Coumestrol was obtained from Kodak Eastman (New York), and genistein and daidzein were provided by Tokyo Kasei (Tokyo, Japan). Equol was synthesized as described by Wessely and Prillinger (*3*) and Lamberton et al. (*4*). The purity of equol was >99% according to GC/MS analysis. Estrogen receptors α and β were provided by Mobitec (Goettingen, Germany).

Surface Plasmon Resonace Analysis and Immobilization of ERE to the Sensor Chip. The Biacore technology uses the optical phenomenon surface plasmon resonance (SPR) to monitor biomolecular interactions in real time. The SPR signal is continuously recorded in a "sensorgram" which presents a picture of the interaction between the immobilized molecule and molecules passing over it. A change in the mass concentration of a macromolecule that is immobilized on the sensor chip surface due to binding of a ligand results in a change of this resonance angle, which is finally measured in resonance units (RU).

For immobilization of the ERE to the CM5 sensor chip, we used HBS-EP buffer (0.01 M HEPES (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid) pH 7.4, 0.15 M NaCl, 0.005% Surfactant P20, 3mM Na_2EDTA) as flow buffer at a flow rate of 5 μL/min. Coupling of streptavidin to the sensor chip was done according to the instructions of the amine coupling kit. The surface was activated by injection of 35 μL of a 1:1 mixture of 0.2 M *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide and 0.05 M *N*-hydroxysuccinimide. By injection of 35 μL of a solution containing 300 μg/mL SA in an acetate-buffer (pH 5.0), a surface of about 3500 RU of immobilized SA in each flow cell was gained. With the injection of 35 μL of ethanolamine, the surfaces were inactivated. The biotinylated oligonucleotides were dissolved in distillated water, diluted to a concentration of 1 μM, then heated to 95 °C for 5 min, and cooled overnight at room temperature. Because estrogen receptors bind more effectively to double-stranded ERE than to single-stranded ERE, we used oligonucleotides which form hairpin structures. In one flow cell a nonspecific 76-bp oligonucleotide without an ERE with the following sequence was immobilized: 5′-AGC TCT TTG ATC TTG ATC GAA CTA CTC GAA CTT ACT CCC CCC GAG CAA GTT CGA GCA GTT CGA TCA AGA TCA AAG A-3′-biotin. In the other flow cell a specific 76-bp oligonucleotide containing the ERE with the following sequence was immobilized: 5′-AGC TCT TTG ATC AGG TCA CTG TGA CCT GAA CTT ACT CCC CCC GAG CAA GTT CAG GTC ACA GTG ACC TGA TCA AAG A-3′-biotin. A 20 μL amount of these oligonucleotides (1 μM) was then injected separately to each flow cell, achieving surfaces of about 1000 RU of immobilized oligonucleotides.

Incubation and Measurement. The estrogen receptors were thawed at room temperature and then stored on ice until measurement. A solution containing 50 nM ER was incubated for 15 min on ice with 17β-estradiol or the phytoestrogens in varying concentrations and then injected over the sensor chip for 360 s at a buffer flow rate of 10 μL/min and a temperature of 25 °C. The sample was addressed serially to the two flow cells, first passing the specific surface and then the nonspecific one. As flow buffer we used 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Tween 20, which was filtered and degassed. The surface was regenerated by injection of 10 μL of a solution containing 50 mM NaCl and 0.5 M NaOH.

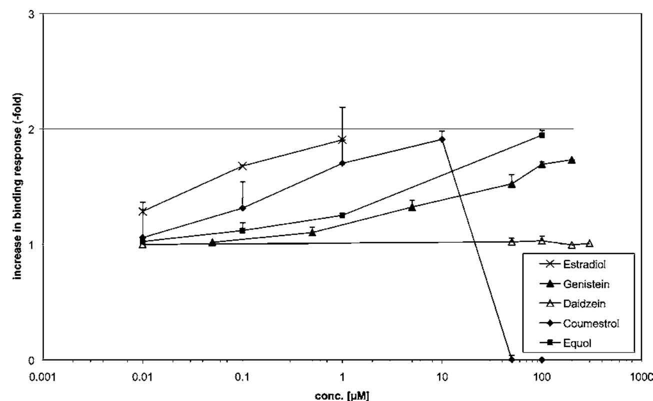

Figure 2. Overview of the relative binding increase of ERα by different phytoestrogens. ERα (50 nM) was incubated for 15 min on ice with different concentrations of 17β-estradiol or phytoestrogens ranging from 0.01 to 300 μM in a solution containing 0.2% DMSO. The response of each incubation after 360 s was divided by the response of the pure estrogen receptor after 360 s in order to get a relative binding increase. This was plotted against a logarithmically scaled concentration for each phytoestrogen. Data are presented as mean ± SD ($n \geq 3$).

RESULTS

ERα. Injection of commercially available ERα over the surface of sensor chip with immobilized oligonucleotide containing an ERE resulted in effective binding even in the absence of a ligand like 17β-estradiol (data not shown). No detectable binding of ERα to the surface of sensor chip with a nonspecific surface was observed.

Binding of unliganded ERα to ERE is in line with the observations of Jisa et al. (*7*) and Cheskis et al. (*8*). These and our observations also confirm the suggestion that in vivo inhibitors such as heat shock proteins can mask the DNA binding domain of the receptor and inhibit binding of unliganded ER to the ERE because commercially available ERα is nearly free from additional proteins (purity > 80%). Incubation of ERα with 17β-estradiol caused an increase in the binding rate in a concentration-dependent manner (**Figure 2**). At a concentration of 1 μM of 17β-estradiol, a saturation level was reached and further increase of the concentration of 17β-estradiol did not raise the binding efficacy. The concentration-dependent effects of 17β-estradiol and the phytoestrogens coumestrol, genistein, daidzein, and equol on the binding response of ERα to ERE are shown in **Figure 2**. For phytoestrogens, the saturation effects were not detected because of the limited solubility of these compounds in buffer containing 0.2% DMSO. To compare the

Table 1. Concentration of Compounds Inducing an Increase in the Binding Rate of ER to ERE by 50 %, the normalized Value of Binding Efficacy, and Relative Binding Affinities of Ligands for Estrogen Receptors α and *β*

	estrogen receptor α			estrogen receptor *β*		
compd	EC(50) (μM)	normalized EC(50) value	normalized binding affinity[a]	EC(50) (μM)	normalized EC(50) value	normalized binding affinity[a]
17*β*-estradiol	0.03	100	100	0.01	100	100
coumestrol	0.2	15	20	0.025	40	140
equol	3.5	0.86		0.4	2	
genistein	15	0.2	4	0.03	25	87
daidzein	>300	<0.01	0.09	0.35	3.3	0.5

[a] Data from Kuiper et al. (*10*).

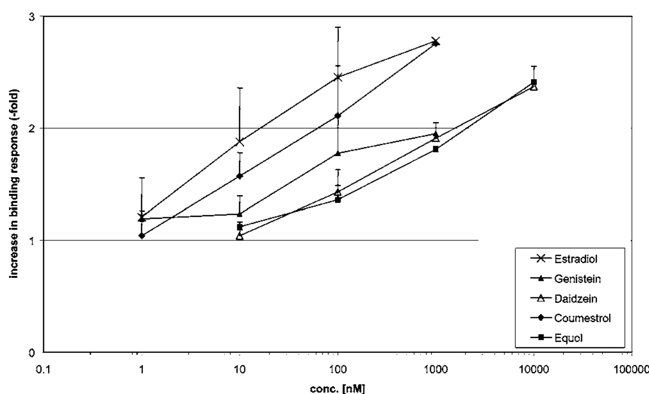

Figure 3. Relative binding increase of ER*β* by different phytoestrogens. ER*β* (50 nM) was incubated for 15 min on ice with different concentrations of 17*β*-estradiol or phytoestrogens ranging from 1 nM to 10 μM in a solution containing 0.2% DMSO. To estimate the relative binding increase, the response after incubation with a compound tested was divided by the response of the estrogen receptor incubated in the absence of an estrogen. Data are given as means ± SD ($n \geq 3$).

efficacy of 17*β*-estradiol and phytoestrogens to induce an increase in the binding rate of ERα to ERE, we estimated the concentration of these compounds causing an increase in the binding rate by 50% as compared to unliganded ER (**Table 1**). 17*β*-Estradiol induced an increase in binding response at lower concentrations than phytoestrogens, indicating the highest efficacy to activate binding of the ER to ERE. The sequence for the efficacy to increase binding was 17*β*-estradiol > coumestrol > equol > genistein > daidzein. Furthermore, it is notable that coumestrol at concentrations higher than 50 μM inhibited binding of the receptor to the ERE strongly.

ER*β*. Similar to the results of ERα, unligated ER*β* also bound to the ERE and this binding response was increased by incubation of ER*β* with 17*β*-estradiol (data not shown). **Figure 3** shows the concentration dependence of the effects of 17*β*-estradiol and phytoestrogens on the binding rate of liganded ER*β* to ERE. For 17*β*-estradiol, the highest activity was detected. At concentrations of about 1 μM, a saturation level was reached. Coumestrol led to the highest increase of the binding response at a concentration of 1 μM. Higher concentrations of coumestrol caused lower response values (data not shown). However, in contrast to the results for ERα, coumestrol did not completely inhibit binding of ER*β* to ERE at these concentrations. Similar to the observations with ERα, the saturation effects were not detected for the other phytoestrogens because of the limited solubility of these compounds in buffer containing 0.2% DMSO. Regarding the efficacy of activation of ER*β*, the sequence was as follows: 17*β*-estradiol > coumestrol > genistein > daidzein > equol.

DISCUSSION

Here we show that dietary phytoestrogens which bind to ERα and ER*β* are also able to increase binding of these receptors to ERE. 17*β*-Estradiol and phytoestrogens tested here showed a greater activation of binding to ERE for ER*β* than for ERα (**Table 1**). For example, daidzein did not cause any increase in the binding of ERα to ERE tested up to a concentration of 300 μM. However, daidzein induced effectively an increase in binding of ER*β* to ERE by 50% at a concentration of 350 nM. Such plasma concentrations of daidzein can be reached in humans by consumption of soy products or extracts, e.g., consumption of an enriched extract from soy germ (1 mg of isoflavonoids/kg of bodyweight) resulted in daidzein plasma c_{max} of 3.3 μM (*9*). This indicates that daidzein can activate ER*β* but not ERα.

The ratios EC(50)α/EC(50)*β* were calculated to be for 17*β*-estradiol, 3; coumestrol, 8; equol, 8.8; genistein, 500; daidzein > 850. These ratios indicate that genistein and daidzein preferentially activate binding of ER*β* to ERE. Such a preferential effect was not that pronounced for neither the endogenous hormone 17*β*-estradiol nor the phytoestrogen coumestrol, and the daidzein metabolite equol and the ratios are comparable. Interestingly, equol, a daidzein metabolite, induced binding of ERα to ERE at least 85 times more effective than daidzein. Thus, the effect of daidzein can be changed from a specific activator of ER*β* to an activator of both ER isotypes α and *β* in humans who are able to convert daidzein to equol.

The binding affinities of compounds tested for ER correlated well with the phytoestrogen-induced increase in binding of ERα to ERE. For ER*β*, there is a distinct difference, especially for daidzein, indicating that ligand-induced dimerization and binding to ERE is not solely dependent on the binding affinities of compounds for ER, and the ligand can also affect this process.

It has been shown that binding of estrogens to the ER induces dimerization of two receptor molecules (*2*). As the SPR is a mass-sensitive phenomenon (*5*, *6*), an increased binding response could be due to binding of a molecule with a 2-fold higher mass (an ER dimer) to the sensor chip. In this case, the maximum response would be a 2-fold increase of the binding rate. In fact, for ERα, the saturation is reached at about a 2-fold increase of the binding response as compared to unliganded ERα. This observation indicates that the binding affinity of ERα for the ERE is not altered by estrogens. The maximum increase in binding for the ER*β* was about 2.7-fold (**Figure 3**). It appears that estrogens are able to induce dimerization of the receptor and also increase the binding affinity of activated ER*β*.

In summary, these data show that dietary phytoestrogens exhibit different efficacy to induce ERα and ER*β* binding to ERE at physiologically relevant concentrations, and this ability can differ from the binding affinities of these phytoestrogens to ERs.

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