Interaction of Phytoestrogens with Estrogen Receptors \( \alpha \) and \( \beta \)

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The human estrogen receptor (hER) exists as two subtypes, hER \( \alpha \) and hER \( \beta \), that differ in the C-terminal ligand-binding domain and in the N-terminal transactivation domain. In this study, we investigated the estrogenic activities of soy isoflavones after digestion with enteric bacteria in competition binding assays with hER \( \alpha \) or hER \( \beta \) protein, and in a gene expression assay using a yeast system. The estrogenic activities of these isoflavones were also investigated by the growth of MCF-7 breast cancer cells.

Isoflavone glycoside binds weakly to both receptors and estrogen receptor-dependent transcriptional expression is poor. The aglycones bind more strongly to hER \( \beta \) than to hER \( \alpha \). The binding affinities of genistin, dihydrogenistin, and genistein, and the two receptors, are equal to the binding affinity of 17 \( \beta \)-estradiol. Equol induces transcription most strongly with hER \( \alpha \) and hER \( \beta \). The concentration required for maximal gene expression is much higher than expected from the binding affinities of the compounds, and the maximal activity induced by these compounds is about half the activity of 17 \( \beta \)-estradiol. Although genistin binds more weakly to the receptors and induces transcription less than does genistein, it stimulates the growth of MCF-7 cells more strongly than does genistein.

Key words isoflavone; human estrogen receptor (hER) \( \alpha \), \( \beta \); isoflavone binding to hER; hER-dependent gene expression; hER-dependent MCF-7 cell growth

Estrogens play important hormonal roles in all vertebrates. Animal estrogens are exclusively steroidal compounds, and the principal physiological estrogen in most species is 17 \( \beta \)-estradiol. Many plants produce compounds that possess estrogenic activity in animals and are thus called phytoestrogens.

Among the foods consumed by humans, soybeans contain the highest concentration of isoflavones. These soy isoflavones (e.g., daidzin, genistin, and glycitin) may have some health-enhancing properties such as prevention of certain cancers,\textsuperscript{1} lowering the risk of cardiovascular diseases,\textsuperscript{2} and improvement of bone health.\textsuperscript{3} The estrogenic activities of these isoflavones may play an important role in their health-enhancing properties. Soy isoflavones have been reported to bind to estrogen receptors and prevent cell growth in breast cancer cells.\textsuperscript{4,5}

We have systematically examined the metabolism of soy isoflavones by enteric bacteria and identified these metabolites.\textsuperscript{6}

Two estrogen receptors (ERs) have been identified to date\textsuperscript{7,8} and the physiological responses to estrogen are known to be mediated within specific tissues by at least these two receptors. The ERs are a 3 A member of the nuclear hormone receptor family and act as ligand-activated nuclear transcription factors.\textsuperscript{9}

In this paper, we examined the estrogenic activities of the isoflavone metabolites by (A) their binding to hER \( \alpha \) and \( \beta \), (B) their effect on estrogen receptor-dependent transcriptional expression, and (C) their effect on the growth of MCF-7 cells, which requires estrogen for growth.\textsuperscript{10,11}

MATERIALS AND METHODS

Chemicals

17 \( \beta \)-Estradiol, Diethylstilbestrol (DES), Bisphenol A (Bis A) and Nonylphenol (NP) were purchased from Sigma Chemical Co. [2,4,6,7-\( ^3 \)H(N)]-17 \( \beta \)-Estradiol (72 Ci/mmol) was purchased from Dai-Ichi Pure Chemicals Co., Ltd. MCF-7 cells were purchased from Dainippon Pharmaceutical Co., Ltd.

Isoflavones

Soybean isoflavones were digested by enteric bacteria and the structures of these digests were determined and isolated as reported.\textsuperscript{9} The products, which are examined in this paper, are shown in Fig. 1.

Preparation of the Extract of Human Estrogen Receptor \( \alpha \) and \( \beta \)

hER \( \alpha \) cDNA was isolated from pBacPAK9/HEG0 kindly supplied by S. Kato\textsuperscript{12} by digestion with \textit{Bam}HI and \textit{XhoI}. hER \( \beta \) cDNA was isolated from pGEX-4T-2-hER \( \beta \)\textsuperscript{13} by digestion with \textit{Bam}HI and \textit{XhoI}. These fragments were ligated into the \textit{Bam}HI/\textit{XhoI} sites of the baculovirus donor vector pFastBac 1 (Life Technologies, Gaithersburg, MD, U.S.A.). Recombinant baculoviruses were generated using the BAC-TO-BAC expression system (Life Technologies) in accordance with the manufacturer’s instructions. The recombinant baculoviruses were amplified and used to infect SF21 cells (Clontech, Palo Alto, CA, U.S.A.). Infected cells were incubated at 28°C and harvested 72 h post infection by centrifugation. The cells were suspended in buffer containing 40 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.2 M KCl, 10% glycerol, 1 mM DTT, and 1 mM PMSE. The extracts were prepared by sonication (10×2). The supernatants of the extracts after centrifugation (15000 rpm×10 min) contained ca. 6 mg/ml protein and were used as hER \( \alpha \) and \( \beta \). The concentrations of hER \( \alpha \) and \( \beta \) were measured using purified hER \( \alpha \) and \( \beta \) purchased from Takara Shuzo.
Co., Ltd. as a standard. Their concentrations were 0.6% and 0.3% of the total protein, respectively. These receptors were stable at -80°C for several months.

**Competition Assay of Isoflavone Estrogen binding to hERα or β**

**Construction of Yeast Strain Carrying Full-Length hERα or β**

Saccharomyces cerevisiae Y190 (MATα, ura3-52, his3-D200, ade2-101, trp1-901, leu2-3, 112, gal4Δgal80D, URA3:: GAL-lacZ, cyhr2, LYS2::: GAL-HIS3) which carries pGBT9-ratER and pGAD424-hTIF2 was kindly supplied by Nishikawa.14) We substituted hERα or hERβ for ratER. pGBT9-ratER-LBD was digested with EcoRI, and then the cleaved open ends were treated with S1 nuclease. The digest was further digested with BamHI followed by treatment with a Klenow fragment and closed by ligation. The plasmid was redigested with BamHI and SalI. pGBT9-hERα or pGBT9-hERβ was prepared by inserting a fragment containing full-length hERα or hERβ obtained by the digestion of pBacPAK9/HEG0 or pGEX4T-2-ER β at BamHI and Xhol sites into the sites obtained by the digestion of pGBT9 by BamHI and SalI (Fig. 2).

**Estrogen Receptor-Dependent Transcriptional Expression Induced by Isoflavone**

The effect of isoflavones on the estrogen receptor-dependent transcription of β-galactosidase in yeast was examined following the methods described by Nishikawa and his colleagues.14) Yeast cells carrying hERα or β were constructed as described by these same authors.14)

**Growth of MCF-7 Cells**

MCF-7 cells were grown in phenol red-free DMEM (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.), penicillin and streptomycin (Gibco BRL, Grand Island, NY, U.S.A.). Cells were grown as a monolayer under these conditions. Cells were harvested as needed for use in experimental trials by trypsinization (0.05% trypsin, 0.53 mM EDTA-4Na; Gibco) to yield a suspension of cells for plating in 96-well tissue-culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were plated at a concentration of 2 × 10⁴ cells/well in phenol red-free DMEM supplemented with 5% heat-inactivated dextran/charcoal-stripped FBS (Hyclone, Logan, UT, U.S.A.) for 24 h prior to the addition of phytoestrogen. The growth of the cells was measured by a sulforhodamine B (SRB) assay15) after 5 d incubation.

**RESULTS**

**Estrogenic Activities of Isoflavones**

DES, Bis A, and NP are known to bind ER, induce transcription and stimulate the growth of MCF-7 cells.16–18) We used these compounds as controls. The results are shown in Fig. 3. DES binds both hERα and β almost as strongly as 17β-estradiol. Bis A and NP bind hERβ better than α. The concentration required to bind 50% is about 10⁴ times greater for Bis A or NP than it is for 17β-estradiol. Tamoxifen which is known as an estrogen
antagonist binds both hER α and β but does not induce transcription. Testosterone did not bind to these receptors confirming their specificity. We also confirmed that 17β-estradiol did not bind to the cell extract prepared from the cells infected by vector baculovirus (data not shown).

In the experiments of estrogen receptor-dependent transcriptional expression, DES induces as efficiently as 17β-estradiol. The concentration required for the maximal induction is about 10^4 times greater for Bis A or NP than it is for 17β-estradiol. These drugs stimulate the growth of MCF-7 cells as shown in Fig. 3. We confirmed that 17β-estradiol did not induce transcription with the cells carrying vector plasmid pGBT9 (data not shown).

The estrogenic activities of the isoflavone derivatives...
shown in Fig. 1 were examined and are shown in Figs. 4, 5, and 6. Figure 4 shows the results obtained for the glycosides: daidzin (DI), glycitin (GLI) and genistin (GI); Fig. 5 shows the results obtained for daidzein (DE), glycitein (GLE) and genistein (GE); and Fig. 6 shows the results obtained for equol (EQ), dihydroglycitein (DGL), and dihydrogenistein (DGE). The results show that glycosides bound poorly to both ERα and ERβ, and induced transcription poorly. The aglycones generally bind to and induce transcription better with hERβ than with hERα. Genistein is the strongest in binding. Though genistein binds ERα as strongly as 17β-estradiol, it does not induce transcription as strongly as 17β-estradiol or DES. Dihydrogenistein binds and induces transcription as efficiently as genistein. The binding of equol is similar to that of genistein and equol is the strongest among these compounds in inducing transcription, especially with hERα. The activity of daidzein is poor. Glycitein binds and induces transcription but the activities of glycitin derivatives are the poorest among these compounds.

The compounds that induce transcription generally stimulated the growth of MCF-7 cells. Genistin, a glycoside of genistein, however, stimulated the growth of cells better than genistein, even though genistin binds to the receptors more weakly and is less effective in inducing transcription than genistein.

**DISCUSSION**

Estrogens are critical to the functioning and maintenance of a diverse array of tissues and physiological systems in mammals. The physiological responses to estrogen are known to be mediated within specific tissues by at least two estrogen receptors (ERs), ERα and ERβ. Studies of the tissue distributions and expression patterns of these receptors indicate that ERα has a broad expression pattern, whereas ERβ has a more focused pattern, with high levels in the ovary, prostate, epididymis, lung and hypothalamus.20,21) The effects of disruption of the ERα gene in ERα knockout mice include an absence of breast development in females and infertility caused by reproductive tract, gonadal and behavioral abnormalities in both sexes.21—26) On the contrary, mice lacking ERβ develop normally.27) Recently, a mouse lacking both ERα and ERβ was constructed.28) Both sexes of this mouse are infertile, but they seem to grow normally and exhibit normal reproductive tract development.

Isoflavones are known to have estrogenic activity.29) We are interested in their activities on ERα and ERβ. Isoflavones contained in soybeans were digested by enteric bacteria. The estrogenic activities of the isolated digests were examined with respect to their binding to hERα and hERβ, estrogen receptor-dependent transcriptional expression, and growth of MCF-7 cells.
Genistein binds to hER β with almost the same efficiency as 17 β-estradiol, but the concentration required to induce transcription is 10^4 times greater for genistein than it is for 17 β-estradiol. Even if genistein bound as efficiently as 17 β-estradiol, the structural transformation of hER β induced by genistein would not be sufficient to facilitate the binding of a coactivator. The induction of transcription by ERs requires a coactivator.²⁹

The E/F region of ER is the ligand-binding domain. The amino acid sequence of the E/F region of hER α is quite different from that of hER β in region E.¹³,³¹,³² This difference is probably responsible for the difference of the binding affinities of the isoflavones for hER α and β.

Pike et al.³³ studied the structure of the ligand-binding domain of hER β in the presence of genistein. They found 3'-OH of genistein corresponds to the 17-OH of 17 β-estradiol and 4'-OH of genistein corresponds to 6-OH of the sterol.

Epidemiological studies suggest that genistein and daidzein reduce the risk of breast and prostate cancers. Although our studies show that not only genistein and daidzein but also equol and glycitein stimulate the growth of MCF-7 though our studies show that not only genistein and daidzein but also equol and glycitein stimulate the growth of MCF-7 cells, the concentrations of these compounds required for cell growth are much higher than the concentration of 17 β-estradiol that is needed. The much higher concentrations required for stimulating cell growth than for binding may explain why these compounds help to reduce the risk of cancer. The preferential expression of ER β in breast and prostate³⁴ and the preferential binding of isoflavones to ER β may explain why these compounds reduce the risk of cancers in these organs. This hypothesis could be tested by determining whether the incidence of breast cancer is reduced by isoflavones in mice lacking ER β (no reduction in breast cancer would be expected in such mice).

It has been reported that intake of isoflavone reduced the serum concentration of estradiol by feedback regulation, and that genistein inhibited tyrosine kinase, which is involved in the cell cycle.³⁵—³⁷ These functions of isoflavone will also help to reduce the risk of cancer.

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