Research Article

# Phytoestrogens activate estrogen receptor β1 and estrogenic responses in human breast and bone cancer cell lines

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Plant-derived phytoestrogens and estrogens in hormone replacement therapies have overlapping yet sometimes divergent effects on the incidence of breast cancer and osteoporosis. Using human MCF-7 breast carcinoma and G-292 osteosarcoma cell lines, it was investigated whether the phytoestrogens genistein and daidzein affect reporter gene transcription *via* the estrogen receptors (ERs) ERα and ERβ1 as well as whether they affect the expression of estrogen-responsive genes in MCF-7 cells and the secretion of the cytokine IL-6 from G-292 cells. The results showed that genistein and daidzein potently trigger transactivation with ERβ1 from estrogen response element-reporter genes (EC50s of 1.7 – 16 nM) although they were 400- to 600-fold less potent than 17β-estradiol (E2) (EC50 of 0.02 – 0.04 nM). E2 was the only potent activator of ERα (EC50 of 0.1 – 0.4 nM). The rank order potency (E2 > genistein > daidzein) is maintained in MCF-7 cells as well as G-292 cells with both receptor subtypes, with a strong receptor selectivity of the phytoestrogens for ERβ1 over ERα. Genistein and daidzein increased the expression of estrogen-responsive genes in MCF-7 cells. Daidzein, like E2, inhibited IL-1β- and hormone-mediated IL-6 secretion from G-292 cells. The results provide a basis for understanding how dietary phytoestrogens protect bone without increasing the risks for breast cancer.

**Keywords:** Daidzein / Estrogen receptors / Genistein / Phytoestrogens

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## 1 Introduction

Phytoestrogens are plant-derived substances with estrogenic activity. Phytoestrogens have potential therapeutic roles in osteoporosis and hormone-dependent cancers [1]. Structurally, phytoestrogens resemble 17β-estradiol (E2) and molecular studies have shown that phytoestrogens can bind to and activate intracellular estrogen receptors (ERs) [2]. The major classes of phytoestrogens include isoflavones, coumestans, and lignans. Isoflavones such as genistein and daidzein are found abundantly in soybeans and soy-based food products.

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**Abbreviations: CAT,** chloramphenicol acetyltransferase; **E2,** 17β-estradiol; **ER,** estrogen receptor; **ERE,** estrogen response element; **ET-1,** endothelin-1; **PTH,** parathyroid hormone 1 – 34

Epidemiological studies have shown that peoples of Asian cultures consuming diets rich in soy, particularly during their younger years, have lower rates of osteoporosis, menopausal symptoms, cardiovascular disease, and select endocrine cancers when they get older compared to peoples of Western cultures [3, 4]. The recent implications of hormone replacement therapy in increasing the risk of heart disease and breast cancer have turned many women toward natural nutritional alternatives including soy phytoestrogens for the relief of menopausal symptoms and for preventive therapy against osteoporosis [5]. Phytoestrogens are effective in postmenopausal women in diminishing moderate to severe hot flushes, night sweats, and vaginal dryness [6, 7].

Epidemiological studies have consistently shown a substantial reduction in breast cancer risk among women with a high dietary intake of phytoestrogens [8]. Phytoestrogen-containing foods such as tofu have been associated with protective effects against breast cancer in pre- and postmenopausal women [3]. Direct studies with genistein and daidzein link phytoestrogen intake with reduced risk of premenopausal breast cancer [9].

Diets rich in phytoestrogens also promote bone-sparing effects. A review of over 60 studies concludes that phytoestrogen-rich diets have positive effects on bone density and fracture rates although the exact mechanism of these actions is unclear [10]. Eight of 12 epidemiological studies show a positive association of soy intake and bone mass, with increases of 6 – 14% in bone mineral density in the highest soy intake group. High consumption of soy and soy isoflavones attenuates bone loss from the lumbar spine of estrogen-deficient perimenopausal women. Soy isoflavone consumption is associated both with significantly higher hip and total body BMD and with attenuated loss of bone mineral content in menopausal and postmenopausal women [11 – 13].

The estrogenic activity of phytoestrogens is dependent upon their interaction with ERα or ERβ. In most systems, the relative binding affinities of genistein and daidzein for ERβ are greater than that for ERα, while E2 binds to ER types with approximately equal affinities [2]. At nutritionally relevant doses, phytoestrogens may selectively interact with ERβ and thus affect only the expression of a subset of estrogen-responsive genes. Differential regulation of estrogen-responsive genes by phytoestrogens may also depend upon the relative ratio of ERα and ERβ present within tissue types. Mammary epithelial cells express relatively high levels of ERα and low levels of ERβ [14, 15]; whereas both ERα and ERβ are present in developing human bone, with ERα more highly expressed in cortical bone and ERβ more prevalent in cancellous bone [16]. Expression may vary during cellular differentiation with the expression of ERβ increasing most significantly during bone mineralization [17].

It is hypothesized that soy isoflavones selectively trigger ERβ-dependent gene expression which may be particular to the cell type but also dependent on the relative expression of ERα and ERβ. We have tested this hypothesis by comparing the transactivational potencies of E2 and the isoflavones, genistein and daidzein, on ERα- and ERβ1-dependent transcriptional activity in human MCF-7 breast carcinoma cells and in human G-292 osteosarcoma cells. In addition, the relative expression of ERα and ERβ1 transcripts in MCF-7 and G-292 cells are determined along with two functional outcomes dependent on ER: estrogen-dependent gene expression in MCF-7 cells and IL-6 secretion from G-292 cells.

## 2 Materials and methods

### 2.1 Materials and reagents

Cyclodextrin-encapsulated E2, genistein (4′,5,7-trihydroxyisoflavone), and daidzein (4′,7-dihydroxyisoflavone) were purchased from Sigma-Aldrich (St. Louis, MO). Genistein and daidzein were dissolved in 100% ethanol. Hormones and reagents were added from 1000-fold stock concentrations. Equivalent volumes of vehicle were included as controls and had no effect on cell viability or assay activities. Parathyroid hormone 1 – 34 (PTH), endothelin-1 (ET-1), and IL-1β were purchased from Sigma-Aldrich and dissolved as stocks as recommended. The GL45-ERE-tkCAT reporter vector containing a minimal estrogen response element (ERE) (5′-AGATCACAGTGACCT-3′) was provided by P. Webb. The human ER expression vectors, pcDNA3.1-ERα and pcDNA3.1-ERβ1, were constructed by inserting the human cDNA for ERα and ERβ1 into pcDNA3.1 (Invitrogen). The parent vector was used as control for the total transfected DNA.

### 2.2 Cell culture

Human MCF-7 breast carcinoma cells (ATCC HTB 22) and human G-292 osteosarcoma cells (ATCC CRL 1423) were cultured in McCoy's medium and Eagle's MEM, respectively, with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified atmosphere of 5% $CO_2$ in air. Under experimental conditions, cells were similarly cultured in media containing 10% dialyzed fetal bovine serum containing 15.8 pg/mL E2 (Sigma Chemical, Lot No. 96H9300) resulting in basal E2 concentrations of less than 6.0 pM. Bovine insulin (10 μg/mL) was added to MCF-7 cells.

### 2.3 Transient transfection and transactivation assay

MCF-7 and G-292 cells were seeded ($1-2 \times 10^5$) in 24-well tissue culture plates 18 h prior to transfection in Opti-MEM I with a DNA-lipofectamine 2000 (Life Technologies) mixture. ERα or ERβ1 expression vectors (250 ng) were transfected along with the GL45-ERE-tkCAT reporter vector (250 ng) and the pSV-β-galactosidase control vector (Promega) (125 ng). Cells were incubated for 3 h with the DNA-lipofectamine 2000 mixture and then incubated with their appropriate media containing hormones or other test agents. After 18 h, the cells were harvested and soluble extracts prepared. Extracts were used for spectrophotometric determination of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside as a substrate in a colorimetric assay. After normalization for β-galactosidase activities, chloramphenicol acetyltransferase (CAT) activities in the extracts were determined by incubation at 37°C for 18 h with 3 μL of [$^{14}$C]chloramphenicol (50 – 60 mCi/mmol, NEN Life Science Products), 5 μL of 5 mg/mL acetyl coenzyme A, and 0.25 M Tris-HCl, pH 8.0, in a volume of 120 μL. The acetylated products were phase extracted and subjected to TLC. Results were analyzed using a BioRad Phosphor Imaging and Molecular Imager System. All data were calculated as the mean% of control ± SEM of at least three different experiments performed in duplicate. Zero and 100% transactivation represent activities with vehicle and 3 nM E2.

 

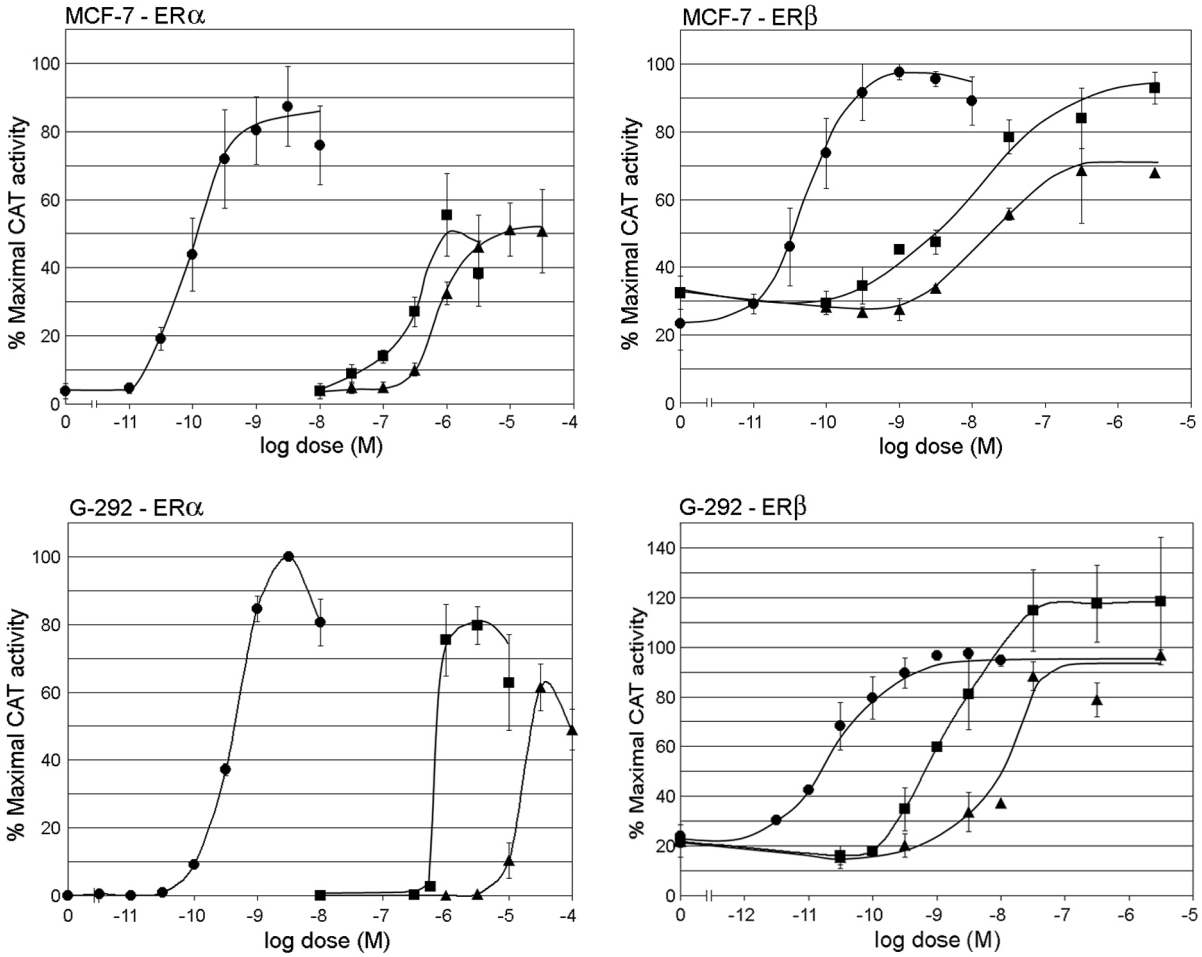


**Figure 1.** E2, genistein, and daidzein transactivation in MCF-7 and G-292 cells expressing human ERα or ERβ1. Cells were transfected with pcDNA3.1-ERα or pcDNA3.1-ERβ1 along with GL45-ERE-tkCAT and pSV-βgal. After 3 h, cells were treated for 18 h with E2 (●), genistein (■), daidzein (▲), or vehicle. After normalization to β-galactosidase activity, CAT activities in cell lysates were assayed. Each data point represents the mean ± SEM of three experiments, each done with duplicate determinations.

### 2.4 Gene array analysis

MCF-7 cells were cultured to 60 – 80% confluency in 75-$cm^2$ tissue culture flasks. Prior to treatment, cells were incubated for 12 h in low serum (0.1%) media. Cells were treated with 1 nM E2, 10 μM genistein, or 10 μM daidzein for 24 h in low serum media. Total RNA was isolated by extraction using TRIzol reagent (Life Technologies). Targeted gene expression profiling was performed using the Human Estrogen-Signaling Pathway GEArray kit (SuperArray). This array system includes housekeeping genes and 23 genes associated with estrogen signaling, including ERs and primary and secondary response genes to estrogen. Results were analyzed by autoradiography using a BioRad Phosphor Imaging and Molecular Imager System. Data were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase expression.

### 2.5 Real-time quantitative RT-PCR

Total RNA was prepared as for gene array analyses. Complementary DNA synthesis kit, SYBR Green, and specific primers for genes of interest were purchased from SuperArray Bioscience. Analyses were performed using the BioRad iQ iCycler and BioRad Gene Expression Macro 1.1. β-Actin was used as an internal control. Values are averages of two independent experiments performed in duplicate.

### 2.6 IL-6 Secretion

G-292 cells were cultured in low E2 media to 60 – 80% confluency in 24-well tissue culture dishes. Cells were pretreated for 24 h with 10 nM E2, 300 nM daiazein, or vehicle. Cells were then washed four times and treated with 10 nM PTH, 10 nM ET-1, 10 pM IL-1β, or vehicle for 18 h.

Supernatants were removed and used to measure IL-6 secretion using the IL-6 ELISA System kit (Amersham Pharmacia Biotech). Results were read on a BioRad #3550 reader using Microplate Manager 4.0 software. Each experimental condition was performed in duplicate from two independent cultures. All data were calculated as the mean% of control.

### 2.7 Statistical analyses

All experiments were performed in triplicate or as indicated. Values are expressed as means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA). Differences between mean values of the treatment groups were tested for significance ($p < 0.05$) using the Student's Newman–Keuls post hoc test. The statistical software package used was ProStat (Poly Software, Pearl River, NY).

## 3 Results

### 3.1 Phytoestrogens stimulate ERE-dependent transactivation in MCF-7 breast carcinoma cells and G-292 osteosarcoma cells

Transient transfection assays were performed to determine whether genistein and daidzein were capable of activating ER-dependent transactivation in two specific estrogen-responsive cell lines, human MCF-7 breast carcinoma cells, and human G-292 osteosarcoma cells. Cells were cultured in reduced estrogen-media (<6.0 pM E2) and transfected with ERα or ERβ1 along with a reporter plasmid containing a minimal ERE in front of the thymidylate kinase transcription initiation sequence and the CAT gene. The ERβ1 subtype was chosen for these studies since RT-PCR analyses showed ERβ1 expression in G-292 cells.

Figure 1 shows the dose–response curves for ERE-dependent transcriptional activation in ERα- and ERβ1-transfected MCF-7 and G-292 cells as a function of E2 or phytoestrogen concentration. E2, genistein, and daidzein stimulated ERE-dependent CAT transcription in MCF-7 and G-292 cells transfected with either ERα or ERβ1. Little or no activity was seen with the parent pcDNA3.1 vector (*i.e.*, without overexpressed ER) suggesting that the response is ER-mediated. The rank order of potency for transcriptional activation is conserved in all cases: E2 > genistein > daidzein. The maximal transactivation responses with either genistein or daidzein were not different from each other but were significantly less than that seen with E2, although this depended on the ER type: 50–80% maximal activity was seen with ERα, whereas 70–120% was seen with ERβ1.

Both the rank order potency of phytoestrogens and strong agonistic activity of genistein and daidzein with ERβ1 in MCF-7 cells are consistent with published reports [18]. However, this is the first report of comparative activities of phytoestrogens and E2 with human G-292 osteosarcoma cells. In G-292 cells, potent transactivational activities of genistein and daidzein (EC50s, 1.7 and 13 nM) were observed with ERβ1 but much less potent activities (EC50s, 655 nM and 15.5 μM) with ERα (Table1).

**Table 1.** Transcriptional activation potencies of E2, genistein, and daidzein in MCF-7 breast adenocarcinoma cells and G-292 osteosarcoma cells transfected with ERα or ERβ1[a)]

| Treatment | ERα EC50 | | ERβ1 EC50 | |
|---|---|---|---|---|
| | MCF-7 | G-292 | MCF-7 | G-292 |
| E2 | 0.11 nM | 0.45 nM | 0.05 nM | 0.03 nM |
| Genistein | 333 nM | 655 nM | 13 nM | 1.7 nM |
| Daidzein | 631 nM | 15.5 μM | 16 nM | 13 nM |

a) EC50 values were derived by nonlinear curve-fitting from the transactivation data of three separate experiments summarized in Fig. 1.

Real-time PCR analyses show that both MCF-7 and G-292 cells express endogenous ERα and ERβ. In MCF-7 cells, there is a 208-fold excess ($p < 0.05$) of ERα mRNA over ERβ mRNA, whereas in G-292 cells there is a 1.3-fold excess (not significant) of ERβ over ERα ($N = 5$).

### 3.2 Phytoestrogens stimulate estrogen-signaling gene expression profiles in MCF-7 breast carcinoma cells

A profile of estrogen signaling from endogenous ERs and other signal transducers can be assessed by analyzing targeted gene expression profiles. Endogenous MCF-7 gene expression driven by E2, genistein, or daidzein was analyzed using human estrogen-signaling pathway GEArrays.

MCF-7 cells were cultured in reduced estrogen-media and then treated with 1 nM E2, 10 μM genistein, 10 μM daidzein, or vehicle for 24 h and total RNA analyzed for gene expression using the human estrogen-signaling pathway arrays. Results are summarized in Table 2. Concentrations at or near maximal for stimulating ERE-dependent gene transactivation *via* ERα or ERβ1 were used. Under these conditions, E2, genistein, and daidzein induced two-fold or greater increases in the estrogen-signaling genes including BRCA1, c-jun, c-myc, cox7RP, cyclin D, and EBAG9. Confirmation of the inductive effects on the estrogen-dependent target EBAG9 was assessed using real-time PCR. Real-time PCR analysis verified the inductive effects of E2, genistein, and daidzein, showing real-time increases of 1.5 ×, 2.7 ×, and 1.8 × with E2, genistein, and daidzein, respectively.

### 3.3 Daidzein and E2 inhibit hormone- and cytokine-stimulated IL-6 secretion from G-292 osteosarcoma cells

E2 inhibits IL-6 secretion from human osteoblasts and bone marrow stromal cells [19, 20]. This action of E2 has been

 

**Table 2.** Effects of E2, genistein, and daidzein on estrogen-signaling pathway gene expression in MCF-7 breast adenocarcinoma cells[a)]

| | E2 | Genistein | Daidzein |
|---|---|---|---|
| β-Actin | 1.16 ± 0.03 | 1.35 ± 0.11[b)] | 1.78 ± 0.13[c)] |
| BRCA1 | 1.68 ± 0.01[c)] | 2.18 ± 0.03[c)] | 2.61 ± 0.04[c)] |
| Cathepsin D | 1.37 ± 0.29 | 1.48 ± 0.15 | 1.32 ± 0.10 |
| c-Jun | 1.97 ± 0.01[c)] | 1.76 ± 0.05[c)] | 2.22 ± 0.07[c)] |
| c-Myc | 8.18 ± 6.15 | 7.26 ± 5.63 | 3.48 ± 2.96 |
| Cox7RP | 2.38 ± 0.16 | 1.79 ± 0.23[b)] | 2.37 ± 0.03[b)] |
| Cyclin D | 5.73 ± 0.81[c)] | 3.41 ± 0.64[c)] | 3.51 ± 0.57[c)] |
| EBAG9 | 2.11 ± 0.32[b)] | 2.09 ± 0.25[b)] | 2.48 ± 0.12[c)] |
| HMG1 | 1.37 ± 0.07[b)] | 1.11 ± 0.04 | 1.71 ± 0.05[c)] |
| Keratin | 1.11 ± 0.08 | 0.83 ± 0.02 | 1.24 ± 0.10 |

a) MCF-7 cells were treated for 24 h with 1 nM E2, 10 μM genistein, 10 μM daidzein, or vehicle. Total RNA was isolated and analyzed for gene expression profiling using the human estrogen-signaling pathway GEArray. Values (means ± SEM, $N = 4$) were normalized relative to GAPDH expression and represent the fold induction over vehicle treatment.
b) $p < 0.05$.
c) $p < 0.01$ *vs.* vehicle-treated cells.

suggested to contribute to the osteoprotective effect of estrogens since, among other evidence, IL-6 knockout mice are protected against the loss of trabecular bone induced by ovariectomy [21]. G-292 cells synthesize and secrete IL-6 in response to inflammatory cytokines and regulatory hormones involved in bone turnover [22]. The effects of E2 and daidzein on basal and stimulated-IL-6 secretion from G-292 cells were determined. G-292 cells were pretreated for 24 h with 10 nM E2, 300 nM daidzein, or vehicle, washed, and then stimulated with 10 nM PTH, 10 nM ET-1, 10 pM IL-1β, or vehicle. After 18 h, cell supernatants were assayed for IL-6 by ELISA (Table 3). In vehicle-pretreated cells, PTH, ET-1, and IL-1β each significantly stimulated IL-6 secretion 1.5-, 2.7-, and 130-fold, respectively, compared to vehicle-stimulated cells. However, in both E2-pretreated and daidzein-pretreated cells, PTH-, ET-1-, and IL-1β-stimulated IL-6 secretion was reduced compared to vehicle-pretreated cells, although the effects of E2 and daidzen on PTH-stimulated secretion did not achieve significance.

**Table 3.** Effects of E2 and daidzein on stimulated-IL-6 secretion from G-292 osteosarcoma cells[a)]

| Stimulus | IL-6 (pg/mL culture supernatant) | | |
|---|---|---|---|
| | Vehicle | E2 | Daidzein |
| Control | 3.2 ± 0.1 | 3.3 ± 0.2[b)] | 3.6 ± 0.7[b)] |
| PTH | 4.7 ± 0.2[c)] | 4.1 ± 0.3[b)] | 4.2 ± 0.2[b)] |
| ET-1 | 8.7 ± 0.4[c)] | 6.8 ± 0.1[d)] | 6.3 ± 0.1[d)] |
| IL-1β | 423 ± 14[c)] | 355 ± 6[d)] | 385 ± 11[d)] |

a) G-292 cells were treated for 24 h with 10 nM E2, 300 nM daiazein, or vehicle, as indicated. After washing, cells were then treated for 18 h with 10 nM PTH, 10 nM ET-1, 10 pM IL-1β, or vehicle for 18 h. IL-6 in the supernatants was quantified using ELISA. Values are means ± SEM ($N = 5–6$).
b) NS *vs.* vehicle treatment.
c) $p < 0.05$ *vs.* control stimulation.
d) $p < 0.05$ *vs.* vehicle treatment.

## 4 Discussion

This study demonstrates the potent agonistic effects of the phytoestrogens genistein and daidzein with overexpressed ERα and ERβ1 in MCF-7 breast carcinoma cells and in G-292 osteosarcoma cells. These activities of phytoestrogens are most likely mediated by their direct activation of ERs as their effects were minimal in the absence of the transfected ER. Although both MCF-7 and G-292 cells contain levels of endogenous ER, responses with ERE reporters in the experiments shown here would be expected to be dominated by the overexpressed ERs.

MCF-7 cells are ER-positive cells, expressing both ERα and ERβ [23, 24]; G-292 cells, although less well characterized, are also ERα- and ERβ-positive cells as shown by real-time PCR. The 200-plus-fold excess of ERα over ERβ in MCF-7 cells is consistent with the ready detection by immunoblot of ERα in nuclear extracts using polyclonal pan-specific polyclonal affinity-purified IgG (Affinity Bioreagents). G-292 cells express approximately equal levels (1.3-fold excess ERβ) of transcripts for ERα and ERβ. RT-PCR and DNA sequencing indicate that the dominant endogenous ERβ in G-292 cells is the ERβ1-long subtype.

Transactivational studies with MCF-7 cells corroborate findings indicating that in this cell line, genistein and daidzein show differential transactivating ability, being significantly more potent (>100 ×) in stimulating ERβ-mediated transcription compared to ERα-mediated transcription [25]. Interestingly, similar studies in human endometrial Ishikawa cells showed genistein-stimulated transactivational potency to be greater with ERβ, but only 20-fold more potent than with ERα[26]. In the present study of MCF-7 cells, genistein and daidzein were 20- to 40-fold more potent in stimulating ERβ-mediated transcription; however, in G-292 cells, the phytoestrogens were 300- to 1000-fold more potent in stimulating ERβ-mediated transcription compared to ERα. Strictly on the basis of EC50 values, the dramatic selectivity for ERβ in G-292 cells is attributed as probably due to a lower potency of genistein and daidzein with the ERα rather than a greater potency with ERβ. This may suggest differential efficacy of coactivator coupling or transcriptional stimulation, and highlights the significance of assaying phytoestrogen transactivation in an osteoblastic cell line. The significance of these synthetic transcriptional effects is highlighted by parallel studies measuring endo-

genous estrogen-mediated effects on two functional responses: gene expression profiles in MCF-7 cells and IL-6 secretion in G-292 cells.

Experiments with human estrogen-signaling pathway oligoarrays and RNA from MCF-7 cells showed that two-thirds of the estrogen targets detected in MCF-7 cells were induced by 1 nM E2. Although these are limited arrays, all the targets that were induced by E2, including BRCA1, c-jun, c-myc, cox7RP, cyclin D, and EBAG9, were also induced by genistein and daidzein, suggesting a common mechanism, that is being ER-mediated. BRCA1, cathepsin D, and EBAG9 have been observed to be directly regulated by E2 through actions of the ER [27]. The ER types mediating the induction cannot be determined from these experiments as data from the transactivation studies show that the concentrations of E2 (1 nM), genistein (10 μM), and daidzein (10 μM) are sufficient to activate both ERα and ERβ in these cells. Although genistein and daizdein potently activate ERβ in these cells, the sole mechanism of gene induction cannot be determined from these experiments.

BRCA1 expression was significantly up-regulated in MCF-7 cells by E2 (1.6 ×), genistein (2.2 ×), and daidzein (2.7 ×). BRCA1 is a tumor-suppressor gene identified as a breast cancer susceptibility gene critical for DNA damage repair and cell cycle checkpoint control. Expression of BRCA1 has been reported to be increased by estrogens in MCF-7 cells and other breast epithelial preparations [28]. It is interesting that in microarray profiling of the effects of phytoestrogens on the expression of estrogen-responsive genes in MCF-7 cells, Ise *et al.* [29] showed an inhibitory effect of E2 on BRCA1 expression with no effect of genistein and daidzein. The present studies used similar concentrations but analyzed the expression at 24 h whereas the Ise *et al.* [29] study looked at the expression at 72 h. Inactivation of BRCA1 in the breast as a result of germ line mutation results in hyperplasia and tumor formation and decreasing BRCA1 levels increased the growth of tumor cells [28]. Induction of BRCA1 expression is associated with apoptosis and reduced tumor cell growth. Phytoestrogen-stimulated up-regulation of BRCA1 as shown here is consistent with phytoestrogens having a protective effect on the breast.

Up-regulation of c-myc is one of the earliest transcriptional responses to E2 in breast cancer cells and has been linked to mitogenesis [30]. Similarly, the inductions of c-jun and cyclin D are part of the mitogenic effect of E2 on MCF-7 cells. Cyclin D1 is strongly induced by E2 in MCF-7 cells and is also associated with ER expression in breast cancer [31]. The strong induction of c-myc, c-jun, and cyclin D by both genistein and daidzain supports their mode of action similar to E2 and dependent on ER.

COX7RP (cytochrome *c* oxidase subunit VII-related protein) and EBAG9 (ER-binding fragment-associated gene 9) were isolated as novel estrogen-responsive genes in MCF-7 cells using an assay based on the binding of the ER-DNA-binding domain to isolated CpG islands enriched in regulatory regions of genes [32]. EBAG9 is an estrogen-responsive gene encoding a tumor-associated antigen expressed at high frequency in a variety of cancers. COX7RP and EBAG9 transcripts are up-regulated by E2 in MCF-7 cells. In the present study, COX7RP and EBAG9 transcripts in MCF-7 cells are increased 2.1- and 2.4-fold by 24 h treatment with 1 nM E2. Expression levels of COX7RP and EBAG9 transcripts were also increased by genistein (1.8- and 2.1-fold) and daidzein (2.4- and 2.5-fold). Real-time PCR analysis of EBAG9 expression confirmed these results, supporting the contention that these phytoestrogens are activating endogenous ERs in MCF-7 cells.

IL-6 is a multifunctional inflammatory cytokine expressed by a variety of normal and transformed cells. In bone, IL-6 is a local regulator of bone turnover and appears to be essential for the bone loss observed under estrogen deficiency [21]. Estradiol activation of ER in bone marrow stromal cells inhibits IL-6 promoter activity and IL-6 production. It is shown here that IL-6 is secreted by G-292 cells in response to the osteotropic hormones PTH and ET-1 and to the inflammatory cytokine IL-1β. Like effects in bone marrow stromal cells, E2 inhibited IL-6 secretion from G-292 cells stimulated by PTH, ET-1, or IL-1β, although the effect on PTH did not achieve statistical significance. Importantly to the hypothesis that phytoestrogens are acting similarly to E2 in osteoblasts, daidzein also inhibited IL-6 secretion induced by ET-1 and IL-1β. The concentration of daidzein used in these experiments, 300 nM, is 20-fold higher than the EC50 for transactivation of ERβ1 (13 nM) and 50-fold lower than the EC50 for transactivation of the ERα (15.5 μM) in G-292 cells, suggesting that daidzein may be acting *via* ERβ to inhibit IL-6 secretion in G-292 cells. These results would suggest that ERβ-specific targeting by dietary phytoestrogens may produce beneficial effects in bone and may be targeted as antiosteoporotic therapy in perimenopausal women. Comparative studies of ERα *versus* ERβ gene knockout studies in mice suggest that ERα may also be involved in the estrogen regulation of serum IL-6 [33].

In summary, this study shows that genistein and daidzein function as phytoestrogens in two separate estrogen-responsive human cell lines, MCF-7 breast carcinoma cells and G-292 osteosarcoma cells. Transactivation studies with transfected ERs, show that both genistein and daidzein are potent activators of ERβ (EC50s 1 – 20 nM) in these cell lines although both are less potent than E2 (EC50s < 1 nM). Comparison with E2-regulated gene expression in MCF-7 cells and E2-inhibition of stimulated-IL-6 secretion from G-292 cells show that both genistein and daidzein mimic these estrogenic activities. The potent stimulation of ERβ-dependent transcription together with the activation of E2-functional responses in MCF-7 and G-292 cells by genistein and daidzein are consistent with the hypothesis that these dietary phytoestrogens may provide potential therapeutic

 

effects in alleviating menopausal symptoms and treating osteoporosis.

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