Estrogen Receptor β-Selective Transcriptional Activity and Recruitment of Coregulators by Phytoestrogens

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Estrogens used in hormone replacement therapy regimens may increase the risk of developing breast cancer. Paradoxically, high consumption of plant-derived phytoestrogens, particularly soybean isolavones, is associated with a low incidence of breast cancer. To explore the molecular basis for these potential different clinical outcomes, we investigated whether soybean isolavones elicit distinct transcriptional actions from estrogens. Our results demonstrate that the estrogen 17β-estradiol effectively triggers the transcriptional activation and repression pathways with both estrogen receptors (ERs) ERα and ERβ. In contrast, soybean isolavones (genistein, daidzein, and biochanin A) are ERβ-selective agonists of transcriptional repression and activation at physiological levels. The molecular mechanism for ERβ selectivity by isolavones involves their capacity to create an activation function-2 surface of ERβ that has a greater affinity for coregulators than ERα. Phytoestrogens may act as natural selective estrogen receptor modulators that elicit distinct clinical effects from estrogens used for hormone replacement by selectively recruiting coregulatory proteins to ERβ that trigger transcriptional pathways.

Estrogens are used in hormone replacement therapy (HRT) to prevent hot flashes, urogenital atrophy, and osteoporosis in postmenopausal women (1, 2). HRT also may prevent heart disease (3), Alzheimer’s disease (4), and colon cancer (5). Unfortunately, HRT has not lived up to its potential to improve the health of women, because estrogens have been associated with an increased incidence of breast (6, 7) and endometrial cancer (8). This relationship has hampered compliance with HRT severely and has sparked an intense pursuit for selective estrogen receptor modulators (SERMs) that have a safer profile (9, 10). Recently, raloxifene has been approved for the prevention and treatment of osteoporosis (11). Raloxifene is classified as a SERM because it exhibits agonist activity in some tissues such as the bone (12, 13) and acts as an antagonist in other tissues including the breast (14). Although these effects are extremely desirable, raloxifene also increases hot flashes (15), is weaker than estrogens at increasing bone mineral density (16), and does not improve cognitive function (17) or prevent hip fracture (13). Thus, the quest for superior SERMs for HRT continues to be intense.

There also is a growing interest in using dietary natural plant estrogens (phytoestrogens), particularly those found in soy products, as a potential alternative to the estrogens in HRT (18). Interest in phytoestrogens has been fueled by observational studies showing a lower incidence of menopausal symptoms, osteoporosis, cardiovascular disease, and breast and endometrial cancers in Asian women who have a diet rich in soy products (19–24). Consistent with epidemiological studies are the findings that soy phytoestrogens prevent mammary tumors (25, 26) and bone loss (27, 28) in rodents and atherosclerosis of coronary arteries in monkeys (29). Soy protein relieves hot flashes in postmenopausal women (30) and attenuates bone loss in the lumbar spine of perimenopausal women (31). Furthermore, a high intake of dietary phytoestrogens is associated with a lower incidence of breast cancer in women (19). Many postmenopausal women are taking phytoestrogens in an effort to alleviate menopausal symptoms without increasing their risk of developing breast cancer. Moreover, many women with a history of breast cancer take phytoestrogens to control menopausal symptoms (32, 33) because estrogens are contraindicated.

The isoflavones, genistein, daidzein, and biochanin A, which are abundant in soybeans (34) and available widely as herbal tablets, are especially popular among postmenopausal women. Despite their popularity and putative health benefits it is clear that we need to know much more about the molecular mechanisms, safety, and efficacy of isoflavones before they can be recommended to postmenopausal women as an alternative to estrogens for HRT. However, it is clearly important to elucidate the molecular mechanisms whereby isoflavones may elicit distinct clinical actions from estrogens used in HRT. Isoflavones have a structure similar to that of 17β-estradiol (E2) and are capable of binding to the two known estrogen receptors, ERα and ERβ (35–37). Compared with ERα, ERβ exhibits a 7–30-fold greater binding affinity for genistein, whereas E2 binds to ERα and ERβ with equal affinity (38, 39). The relatively selective binding of genistein to ERβ indicates that isoflavones may...
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produce distinct clinical effects from estrogens by selectively triggering ERβ-mediated transcriptional pathways or differentially triggering transcriptional activation or repression pathways by ERβ.

To test this hypothesis, we compared the effects of isoflavones and E2 on transcriptional repression and activation in the presence of ERα or ERβ. Our data demonstrate that isoflavones selectively trigger the transcriptional pathways of ERβ, particularly transcriptional repression. In addition to selectively binding to ERβ, our results suggest that the ERβ selectivity of isoflavones involves their capacity to induce an activation function-2 (AF-2) surface of ERβ that has greater affinity for coregulators such as glucocorticoid interacting receptor protein 1 (GRIP1) (40) compared with ERα. Phytoestrogens may act as natural SERMs by selectively recruiting coregulators that trigger ERβ-mediated transcriptional pathways.

MATERIALS AND METHODS

Plasmids—Human ERs and ERβ were provided by P. Chambron and J.-A. Gustafsson, respectively (41). Gal-GRIP1 and GST-GRIP1 were provided by M. Stallcup (42). Three copies of the −125 to −82 human TNF-α promoter fragment (43) or one copy of the ERE from the frog vitellogenin A2 gene (5′-TCAGGTCACTGTAGACCTGTA-3′; vitA2-ERE) were ligated into the polylinker upstream of −32 to +45 herpes simplex thymidine kinase (tk) promoter linked to luciferase (TNF-RE tkLuc and ERE tkLuc, respectively). A synthetic oligonucleotide containing the 17-nucleotide Gal-responsive element (5′-CGCAATGATCCCAAAGTAGACCTGCCCAGACT-3′) or the AP-1-like site (5′-TGAGCTCA-3′) at the −105 to −95 region of the TNF-RE and cloned upstream of the −32 to +45 tk promoter (Gal-TNF-RE tkLuc).

Cell Culture, Transfection, and Luciferase Assays—U937, U2OS, MDA-MB-435, and MCF-7 cells were obtained from the cell culture facility at the University of California, San Francisco. U937 cells were maintained as described previously (44), whereas U2OS, MDA-MB-435, and MCF-7 cells were maintained and subcultured in phenol red-free Dulbecco’s modified Eagle’s medium/F-12 media containing 5% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. For experiments, cells were collected, transferred to a cuvette, and then electroporated with a Bio-Rad gene pulser as described previously (41) using 3 μg of reporter plasmid and 1 μg of ERα or ERβ expression vectors. After electroporation, the cells were resuspended in media and plated at 1 ml/dish in 12-well multilplates. The cells were treated with E2, genistein, daidzein, or biochanin A (Sigma-Aldrich) 3 h prior to exposure to 5 ng/ml TNF-α (R & D Systems) for 24 h at 37 °C. Cells were solubilized with 200 μl of 1× lysis buffer, and luciferase activity was determined using a commercially available kit (Promega). The concentration of hormone required to produce a half-maximal induction (EC50) or inhibition (IC50) of luciferase activity was determined using a commercially available kit (Promega) and [35S]methionine. For each binding reaction, a 2-μl aliquot of translation product was incubated with Escherichia coli-expressed GST-GRIP1 immobilized to glutathione-Sepharose beads (Amersham Pharmacia Biotech) in the presence of vehicle control (0.1% ethanol), E2, or genistein. The samples were rocked gently at 4 °C for 2 h. After extensive washing of the beads, the labeled proteins were eluted with SDS-polycacrylamide gel electrophoresis loading buffer and separated on a 12% SDS-polycrylamide gel. The radiolabeled bound ERs were detected by autoradiography and analyzed using the Storm phosphorimaging system and ImageQuant software (Molecular Dynamics).

RESULTS

Estrogens Selectively Repress the TNF-α Promoter through ERβ—To investigate the effects of isoflavones on transcriptional repression, we used the −125 to −82 region (43) of the TNF-α promoter (TNF-α-responsive element, (TNF-RE)) because this region mediates TNF-α activation and E2 repression (41). E2 produced a profound dose-dependent repression of TNF-α activation of the TNF-RE upstream of a minimal tk promoter (TNF-RE tkLuc) with either transfected ERα (Fig. 1A) or ERβ (Fig. 1B) in U937 cells. Daidzein and biochanin A had no effect on TNF-α activation of the TNF-RE with ERα, whereas genistein produced a minor repression at 1 μM (Fig. 1A). In contrast, all three isoflavones produced a large repression (30–60%) of TNF-α activation of TNF-RE in the presence of ERβ (Fig. 1B). Genistein is the most potent isoflavone and is about 65-fold weaker than E2 at repression (IC50 = 8.5 versus 0.13 nM). The isoflavones are more effective also at triggering transcriptional activation of a classical estrogen response element (ERE) in U937 cells with ERβ (Fig. 2B) compared with ERα (Fig. 2A). However, isoflavones are about 10–300-fold more potent at triggering transcriptional repression compared with transcriptional activation with ERβ (genistein, IC50 = 8.5 nM, EC50 = 55 nM; daidzein, IC50 = 0.072 μM, EC50 = 1.2 μM; biochanin A, IC50 = 0.17 μM, EC50 = 50 μM).

Genistein Decreases TNF-α mRNA in Bone Cells—The effect of genistein on endogenous TNF-α gene expression was investigated in a human osteosarcoma cell line (U2OS) because these cells express ERα and ERβ, as demonstrated by RT-PCR (data not shown), and TNF-α is involved in the pathogenesis of osteoporosis (47). U2OS cells were treated with E2 or genistein for 24 h and then exposed to TNF-α for 1 h. TNF-α produced a profound induction of TNF-α mRNA as determined by RT-PCR that was repressed markedly by E2 or genistein (Fig. 3A). The observation that genistein inhibits endogenous TNF-α mRNA in untransfected cells demonstrates that repression of TNF-α transcription by genistein is physiological and not caused by nonspecific squelching of transcriptional factors by transfected E2 receptors. To determine which ER isomer is responsible for repressing the endogenous TNF-α gene, we transfected U2OS cells with ERα or ERβ. Although the endogenous ERα genes are capable of repressing the native TNF-α gene, they are not present in high enough levels to repress the large number of transfected plasmids containing the TNF-RE. Fig. 3B shows that genistein is very effective at repressing the TNF-RE in cells transfected
with ERβ but not ERα. These results indicate that genistein represses the endogenous TNF-α gene through ERβ even though U2OS cells also express ERα.

Isoflavones Are Weak ERα Agonists in Breast Cancer Cells—Our results indicate that isoflavones selectively promote ERβ-mediated transcription. To explore the activity of genistein on ERα in breast cancer cell lines, we compared the effects of E2 and genistein on ERα activation of ERE tkLuc in an ER-negative breast cancer cell line (MDA-MB-453) stably transfected with ERα and on the proliferation of MCF-7 cells, which express endogenous ERα but not ERβ as determined by RT-PCR (data not shown). Similar to transiently transfected U937 and U2OS cells, genistein is much weaker than E2 at activating an ERE in the ERα MDA-MB-453 stable cells (Fig. 4A) and stimulating the proliferation of MCF-7 cells (Fig. 4B). Thus, genistein is a weak ERα agonist in cells transiently (U937 and U2OS) or stably (MDA-MB-453) transfected with ERα and in cells that express endogenous ERα (MCF-7).

Isoflavones Selectively Recruit GRIP1 to ERβ—A potential explanation for ERβ-selective activity is that isoflavones induce a functional AF-2 surface in ERβ but not ERα because we showed previously that the AF-2 surface is required for repression (41). Consistent with this hypothesis is the observation that an ERβ with a mutation in helix 3 (K314A) of the AF-2 surface failed to promote repression in response to genistein (Fig. 5). Because binding of coregulatory proteins (48, 49) to the AF-2 surface is required for repression by E2 (41), we compared the effects of E2 and genistein on functional interactions that occur at the TNF-RE between the coregulator, GRIP1 (40), and ERα or ERβ. For these studies, a Gal response element was inserted in the center of an AP-1-like site in the TNF-RE (Gal-TNF-RE), which is essential for TNF-α activation and E2 repression (41). Gal-GRIP1 was used for these studies instead of Gal-ER because ERs do not bind directly to the TNF-RE (41).
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**FIG. 3.** A, genistein inhibits endogenous TNF-α gene expression in human osteosarcoma cells. U2OS cells were treated with 10 nm E2, 1 μM genistein (Gen) or ethanol (Cont) for 24 h and then exposed to TNF-α (5 ng/ml) for 1 h. Total RNA was isolated, and the expression of TNF-α mRNA was determined by RT-PCR. Ethidium bromide staining shows a 444-base pair PCR product of the TNF-α gene and a 217-base pair product for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as internal control for the quality of RNA prepared. B, genistein selectively represses transcription of the TNF-RE in U2OS cells through ERβ. U2OS cells were transfected with TNF-RE tkLuc (3 μg) and 1 μg of expression vector for either ERα (●) or ERβ (▲). The cells were treated for 24 h with increasing concentrations of genistein and then assayed for luciferase activity. Each data point represents the mean of triplicate samples. The S.E. was <10%.

but may be tethered to the TNF-RE through coregulators such as GRIP1. Gal-GRIP1 activated Gal-TNF-RE tkLuc ~20-fold (data not shown). E2 is extremely potent at inhibiting Gal-GRIP1 activation of Gal-TNF-RE tkLuc in the presence of either ERα (Fig. 6A) or ERβ (Fig. 6B) (IC50 = 28.5 pm for ERα, and IC50 = 1.5 pm for ERβ). In contrast, genistein is much more potent at repressing Gal-GRIP1 activation with ERβ (IC50 = 49 pm) compared with ERα (IC50 = 1.8 μM). Furthermore, at saturating levels (10 μM), genistein produced a 33% repression with ERα compared with a maximal 72% repression with ERβ at only 10 nm.

These results suggest that genistein creates an AF-2 surface in ERβ that permits the binding of GRIP1 more efficiently compared with ERα. To investigate this hypothesis directly, glutathione S-transferase-GRIP1 pull-down assays were performed with either [35S]-labeled ERα or ERβ in the presence of E2 or genistein. A similar dose-dependent increase in binding of ERα or ERβ to GRIP1 was observed with E2 (Fig. 7A). In contrast, genistein is more effective at enhancing the interaction between GRIP1 and ERβ (Fig. 7B). At 10 μM, binding of ERβ to GRIP1 is 2-fold greater than with ERα. These findings demonstrate that genistein creates an AF-2 surface in ERβ that has a higher affinity for GRIP1 than that in ERα.

**DISCUSSION**

Estrogens in HRT improve menopausal symptoms but are associated with an increased risk of breast (6, 7) and endometrial cancer (8). To overcome the uterotrophic effects of estrogens, women with a uterus are treated also with progesterone in HRT regimens. Unfortunately, the addition of progesterone may increase the risk of breast cancer further (50, 51) and attenuate potential benefits of estrogens on the cardiovascular system (52). The current challenge is to discover estrogens that retain their ability to prevent menopausal symptoms without promoting breast cancer or requiring progesterone for endometrium protection. The development of more ideal estrogens for HRT requires a greater understanding of how different estrogenic compounds differentially regulate gene activation and repression by ERα and ERβ.
We have shown that isoflavones elicit distinct transcriptional actions from estrogens. E2 effectively triggers both ERa- and ERb-mediated transcriptional activation or repression pathways. In contrast, our results demonstrate that isoflavones are weak ERa agonists and potent ERb agonists because they are effective only at triggering transcriptional activation or repression with ERb. The key question is how do isoflavones elicit distinct transcriptional actions from estrogens despite the fact they both bind to the same binding pocket of ERa and ERb (53–55)? One possibility is that isoflavones bind to ERb more effectively than to ERa. In fact, ERb has a 30-fold greater affinity for genistein compared with ERa (39). However, this difference in binding affinity is unlikely to account entirely for the distinct transcriptional actions of isoflavones because we observed that isoflavones were over a 1,000-fold more potent at triggering transcriptional activity with ERb compared with ERa. Furthermore, at saturating levels (10 μM), genistein was less effective at repressing GRIP1 activation of Gal-TNF-RE tkLuc with ERa and recruiting GRIP1 to ERa, compared with ERb. These studies indicate that the divergent transcriptional actions of estrogens and isoflavones probably also result from differences in their ability to recruit coregulators and trigger transcriptional functions of ERa or ERb. These data are consistent with the discoveries that coregulator proteins (48, 49) are required for both transcriptional activation and repression by ERs (41, 56, 57).

E2 nonselectively recruits coregulators to ERa and ERb, whereas isoflavones selectively recruit coregulators to ERb. By recruiting coregulators such as GRIP1 to both ERs, E2 effectively triggers transcriptional activation and repression pathways for both ERa and ERb. Undoubtedly, E2 elicits its full spectrum of beneficial and adverse effects by triggering all transcriptional pathways of ERs. In contrast, at physiological...
levels (0.55–0.86 μM) (58) genistein is very weak at recruiting GRIP1 to ERα, but it is potent at recruiting GRIP1 to ERβ. By selectively recruiting coregulators to ERβ, isoflavones would only trigger ERβ-mediated transcriptional pathways. These results suggest that isoflavones should be effective at eliciting the clinical effects that are mediated by ERβ but not ERα. Moreover, isoflavones are 10–300-fold more potent at triggering transcriptional repression compared with activation. These results indicate that it may be possible to develop transcriptional activation or repression-selective estrogens for HRT. It is unclear why genistein recruits GRIP1 more effectively to ERβ than to ERα. However, the binding of GRIP1 may stabilize the estrogen-ER complex more effectively than the genistein-ERα complex because the binding of coregulators has been shown to slow the rate of dissociation of an agonist from the ER-coregulator complex (59).

The lack of regulation of ERα-mediated genes and the potent repression of ERβ-mediated genes by isoflavones may account for the low incidence of menopausal symptoms, osteoporosis, cardiovascular disease, and breast and endometrial cancer in Asian countries (19, 21–24). For example, our account for the low incidence of menopausal symptoms, osteoporosis, cardiovascular disease, and breast and endometrial cancer in Asian countries (19, 21–24).

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54. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95**, 927–937
55. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engstrom, O., Ljunggren, J., Gustafsson, J. A., and Carlquist, M. (1999) *EMBO J.* **18**, 4608–4618
56. Katzenellenbogen, B. S., Montano, M. M., Ediger, T. R., Sun, J., Ekena, K., Lazennec, G., Martini, P. G., McInerney, E. M., Delage-Mouroux, R., Weis, K., and Katzenellenbogen, J. A. (2000) *Recent Prog. Horm. Res.* **55**, 163–193
57. Klinge, C. M. (2000) *Steroids* **65**, 227–251
58. Barnes, S., Sfakianos, J., Coward, L., and Kirk, M. (1996) *Adv. Exp. Med. Biol.* **401**, 87–100
59. Gee, A. C., Carlson, K. E., Martini, P. G., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (1999) *Mol. Endocrinol.* **13**, 1912–1923
60. Makela, S., Savelainen, H., Aavik, E., Myllarniemi, M., Strauss, L., Tarkinen, E., Gustafsson, J., and Hayry, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 7077–7082
61. Taylor, A. H., and Al-Azzawi, F. (2000) *J. Mol. Endocrinol.* **24**, 145–155
62. Upmalis, D. H., Lobo, R., Bradley, L., Warren, M., Cone, P. L., and Lamia, C. A. (2000) *Menopause* **7**, 236–242
63. Duncan, A. M., Underhill, K. E., Xu, X., Lavalier, J., Phipps, W. R., and Kurzer, M. S. (1999) *J. Clin. Endocrinol. Metab.* **84**, 3479–3484
64. Foth, D., and Cline, J. M. (1998) *Am. J. Clin. Nutr.* **68**, Suppl. 6, 1413–1417