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Phytochemical Glyceollins, Isolated from Soy, Mediate Antihormonal Effects through Estrogen Receptor α and β*

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ABSTRACT

The flavonoid family of phytochemicals, particularly those derived from soy, has received attention regarding their estrogenic activity as well as their effects on human health and disease. In addition to these flavonoids other phytochemicals, including phytostilbene, enterolactone, and lignans, possess endocrine activity. The types and amounts of these compounds in soy and other plants are controlled by both constitutive expression and stress-induced biosynthesis. The health benefits of soy-based foods may, therefore, be dependent upon the amounts of the various hormonally active phytochemicals within these foods. The aim was to identify unique soy phytochemicals that had not been previously assessed for estrogenic or antiestrogenic activity. Here we describe increased biosynthesis of the isoflavonoid phytoalexin compounds, glyceollins, in soy plants grown under stressed conditions. In contrast to the observed estrogenic effects of coumestrol, daidzein, and genistein, we observed a marked antiestrogenic effect of glyceollins on ER signaling, which correlated with a comparable suppression of 17β-estradiol-induced proliferation in MCF-7 cells. Further evaluation revealed greater antagonism toward ERα than ERβ in transiently transfected HEK 293 cells. Competition binding assays revealed a greater affinity of glyceollins for ERα *vs.* ERβ, which correlated to greater suppression of ERα signaling with higher concentrations of glyceollins. In conclusion, we describe the phytoalexin compounds known as glyceollins, which exhibit unique antagonistic effects on ER in both HEK 293 and MCF-7 cells. The glyceollins as well as other phytoalexin compounds may represent an important component of the health effects of soy-based foods. (*J Clin Endocrinol Metab* **86:** 1750–1758, 2001)

FLAVONOIDS REPRESENT a family of phytochemicals that function to deter herbivores, act as antibacterial/antifungal agents, and stimulate the formation of symbiotic relationships with nitrogen-fixing bacteria (1–3). The family of flavonoids is often subclassified into groups of chemicals referred to as flavones, isoflavonoids, chalcones, and coumestans based on their shared structural similarity. Although the functions of these diverse compounds are not completely understood, they not only affect bacteria and fungi, but have been reported to exert effects on mammals as well (1–5). The observations of sheep grazing on fields rich in clover and cheetahs fed high soy diets in zoos have demonstrated that flavonoids and related phytochemicals can affect mammalian health (5–7). Of interest was the observation that these compounds function as estrogenic mimics or phytoestrogens and may represent important dietary factors affecting human health (8–13). The estrogenic phytochemicals, which include flavonoids, lignans, phytostilbenes, and enterolactones, appear to primarily function by binding to and activating the estrogen receptor (ER), albeit at 100-1000 greater concentrations than 17β-estradiol (14–18). Two key constitutive isoflavonoids most often detected in soybean tissue, genistein and daidzein, have been widely examined for these effects. The observation that soy phytochemicals can function as estradiol (E_2) agonists is consistent with the observed health benefits of soy foods, such as decreased incidence of osteoporosis and cardiovascular disease (8–13, 19–23). However, the similar decrease in risk of breast cancer would indicate a potential antiestrogenic activity of soy phytochemicals (17–21). Additionally, the ability of soy isoflavonoids to prevent carcinogen-induced mammary tumorigenesis further demonstrates the potential antiestrogenic effects of these compounds. Consistent with this information, certain phytochemicals have been reported to exert anties-

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trogenic effects at higher concentrations (17, 18). These studies, however, were not exclusive to soy-derived isoflavonoids, suggesting that many flavonoids may function as both ER agonists and antagonists in a dose- and cell type-specific manner. The recent identification of a second estrogen receptor β (ERβ) with different affinity for and *trans*-activation by phytoestrogens represents another mechanism by which flavonoids may function to regulate estrogen signaling (24–26).

The suggestion that the high isoflavonoid content of soy may function to prevent cancer and disease is bolstered by the observation that the predominant isoflavonoids found in soy, genistein and daidzein, can affect estrogen signaling and prevent cancer in animal models. However, genistein and daidzein represent only two compounds in the complex flavonoid biosynthetic pathway, as shown in Fig. 1A, and the amount and type of isoflavonoid present in soy can be readily altered in response to external stimuli. The recent demonstration that the soy isoflavonoid glycitein can function as an estrogen illustrates that other isoflavonoids must be considered in relation to the health effects of soy products (27). Additionally, environmental factors and growth conditions can alter the biosynthesis leading to the production of numerous flavonoids that have not been characterized for their effects in mammalian systems (28–30).

Phytoalexins constitute a chemically heterogeneous group of substances belonging to the various subclassifications of flavonoids mentioned above. Phytoalexins are low molecular weight antimicrobial compounds that are synthesized *de novo* and accumulate in plants as a stress response (4, 31). The phytoalexins are generally lipophilic compounds that are products of a plant's secondary metabolism and often accumulate at infection sites at concentrations that inhibit fungal and bacterial growth (4, 31). Countless stress factors or physical stimuli induce phytoalexin accumulation, including freezing, UV light exposure, and exposure to microorganisms. In addition, compounds referred to as elicitors, either abiotic or biotic, can stimulate the biosynthesis of phytoalexins (4, 28–29, 31–36). Given that the biosynthesis of isoflavonoids, particularly phytoalexins, can be regulated by external factors, the type and amount of hormonally active phytochemicals may vary from source to source. Additionally, the environmentally induced biosynthesis of unique isoflavonoids of undefined hormonal activity may represent an important component of both the beneficial and/or detrimental effects of these compounds on human health.

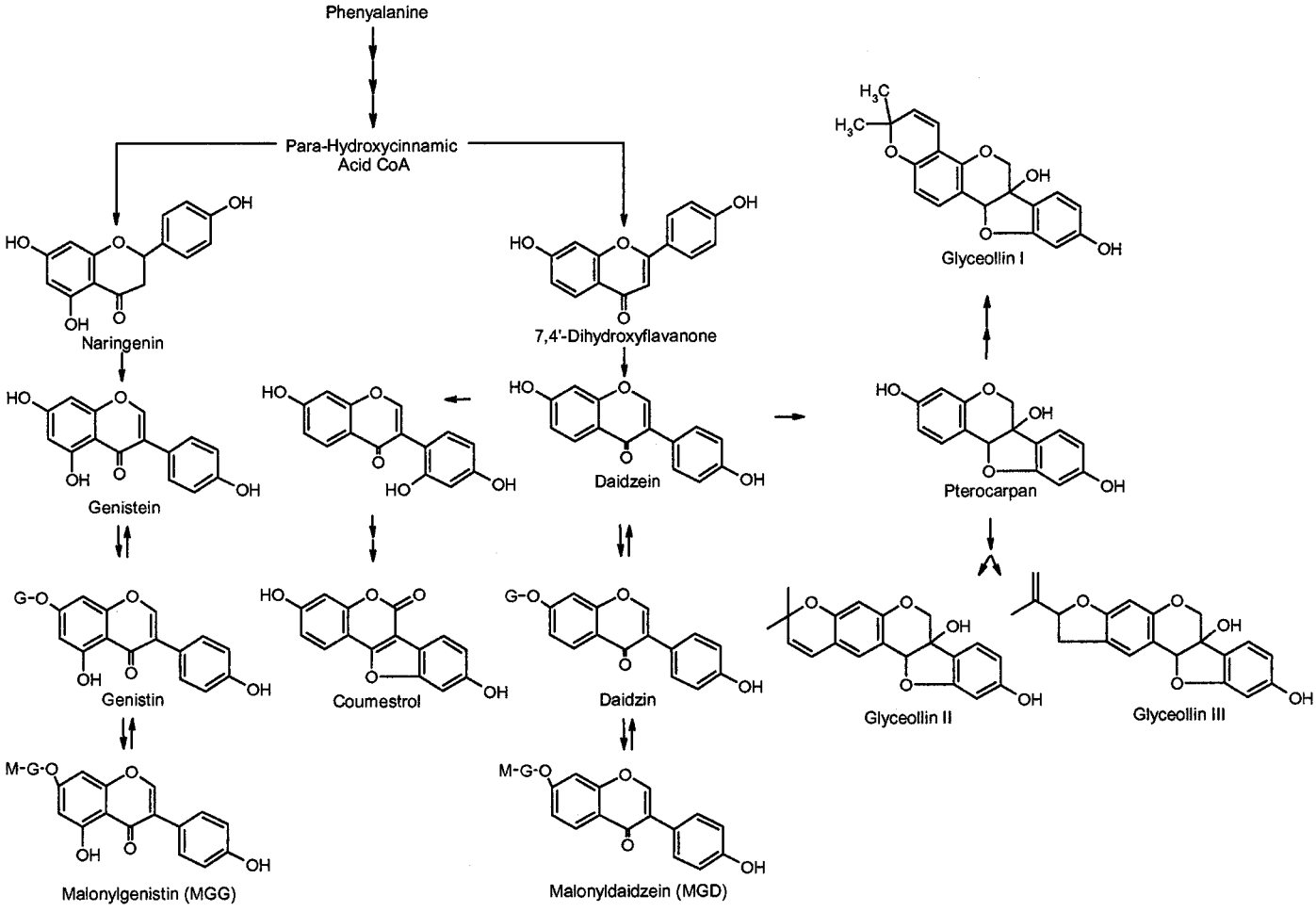

FIG. 1. Flavonoid pathway showing the biosynthetic route from phenylalanine to coumestrol, genistein, daidzein, and the conjugated forms, daidzin, genistin, MGD, and MGG. Also detailed is the biosynthetic pathway from daidzein to glyceollins I–III.

The specific aim of this study was to identify unique soy phytochemicals that have not been previously assessed for estrogenic or antiestrogenic activity and determine whether the altered biosynthesis of flavonoids represents a point of regulation of the hormonal activity of soy products. The present study describes induction of the soybean phytoalexins glyceollins I–III by the fungus *Aspergillus sojae*, a nontoxin-producing *Aspergillus* strain commonly used in the fermentation of soybeans to produce soy sauce and miso. The glyceollins represent a group of phytoalexins whose biosynthesis is increased in response to stress signals. The glyceollin isomers I–III have core structures similar to that of coumestrol and are derived from the precursor daidzein in the glyceollin pathway (see Fig. 1B). The ability of the glyceollins to regulate estrogen signaling was analyzed using the ER-positive MCF-7 human breast carcinoma cell line and ER-negative HEK 293 cells transfected with either ERα or ERβ. Although the glyceollins displayed only slight estrogenic activity, they did cause a dose-dependent suppression of 17β-estradiol-induced *trans*-activation and MCF-7 cell proliferation. The glyceollins also functioned to suppress estrogen activity through both ERα and ERβ, which correlated with binding to ERα and ERβ, respectively. Here we describe the isoflavonoid phytoalexins known as glyceollins I–III, which are synthesized in soy under stress conditions and exhibit a unique antagonistic effect on ER activity in a number of hormone-responsive systems.

Materials and Methods

Chemicals and plasmids

The isoflavonoids daidzein, genistein, and coumestrol were obtained from Indofine Chemical Co. (Somerville, NJ). 4-Hydroxytamoxifen was purchased from Sigma (St. Louis, MO). ICI 182,780 was provided by Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Glyceollins I, II, and III were isolated using a procedure developed in this laboratory. Soybean seeds (50 g) were sliced and inoculated with *Aspergillus sojae*. After 3 days isoflavonoids were extracted from the inoculated seeds with 80% ethanol. The glyceollins were isolated using preparative scale high pressure liquid chromatography (HPLC) and were confirmed by UV-visable spectrophotometry and electrospray mass spectrometry. A mixture of glyceollins I, II, and III in a ratio of 6:2:1 was isolated and used in subsequent analyses. The solvents acetonitrile (HPLC grade) and ethanol were purchased from Aldrich Chemical Co., Inc. (Metuchen, NJ). H_2O treated with a Millipore Corp. system (Bedford, MA) was used during sample preparation procedures and HPLC analyses.

ERβ complementary DNA (cDNA) was provided by Jan-Åke Gustafsson (Karolinska Institute, Stockholm, Sweden) in pBluescript. ERα and ERβ expression vectors were constructed by inserting the ERα and ERβ cDNA, respectively, into pcDNA 3.1 vector (Invitrogen, San Diego, CA). ERα cDNA (2090 bp) was cleaved from plasmid (pBluescript) with *Bam*HI/*Eco*RI and then ligated into the pcDNA3.1. ERβ cDNA (1460 bp) was cleaved from Plasmid (pBluescript) with *Hin*dIII/*Bam*HI and then ligated into the pcDNA3.1. Each construct was verified by detailed restriction mapping.

Soybean treatment and harvesting

A. sojae (SRRC 1125) cultures were grown at 25 C in the dark on potato dextrose agar. After 5 days inoculum was prepared by harvesting conidia (3.4×10^7/mL) in 15 mL sterile distilled H_2O. Buckshot 66 soybean was donated by Louisiana State University Agricultural Center (Baton Rouge, LA). Seeds from commercial soybean variety Buckshot 66 were surface-sterilized for 3 min in 70% ethanol, followed by a quick deionized H_2O rinse and two 2-min rinses in deionized H_2O. Seeds were presoaked in sterile deionized H_2O for 4–5 h before placement into treatment chambers (three seeds per chamber). Each chamber consisted of a petri dish (100 × 15 mm, four compartments); each compartment was lined with two autoclaved filter papers (Whatman, Clifton, NJ) moistened with 0.5 mL distilled H_2O. One seed was placed into a single compartment then sliced in half longitudinally. *A. sojae* spore suspension (10 μL) was applied to the cut surface of each seed. All chambers were stored at 25 C in the dark for 3 days, then transferred to −70 C. Soy extracts were prepared from both *A. sojae*-inoculated and noninoculated 3-day-old seeds. Soy extracts were extracted from 5 g finely ground seeds in 8 mL ethanol and heated at 50 C for 1 h, cooled, then centrifuged at 14,000 × *g* for 10 min. Extracts were filtered through 0.45-μm pore size sterile filter units (Gelman Sciences, Ann Arbor, MI). Stock solutions were prepared as follows. Two milliliters of each extract were evaporated to dryness and dissolved in dimethylsulfoxide at a concentration of 100 mg/mL.

HPLC analyses of phytochemicals

HPLC analyses were performed on a Waters 600E System Controller combined with a Waters UV-visable 486 detector (Waters Corp., Milford, MA). Soy isoflavonoids were extracted from cotyledons (0.3–0.6 g) and homogenized (Tekmar Tissumizer; Tekmar Co., Cincinnati, OH) in 1.5 mL 80% ethanol. Homogenate was heated at 50 C for 1 h, cooled, then centrifuged at 14,000 × *g* for 10 min, and the supernatant was run on HPLC. An aliquot (100 μL) of supernatant was directly analyzed by HPLC. Isoflavonoids were monitored at a wavelength of 260 nm, but the glyceollins were monitored at 285 nm. Separations were carried out using a Multiring C_{18} (4.6 × 250 mm; 5 μm; Vydac, Hesperia, CA) reverse phase column. A guard column containing the same packing was used to protect the analytical column. Elution was carried out at a flow rate of 1.0 mL/min with the following solvent system: A = acetic acid/water (pH 3.0); B = acetonitrile; 0% B to 45% B in 17 min, then 45% B to 90% B in 10 min followed by holding at 90% B for 6 min. Retention times for the isoflavonoids were as follows: daidzin (13.4 min), genistin (15.0 min), malonyldaidzin (MGD; 15.3 min), malonylgenistin (MGG; 16.7 min), daidzein (17.8 min), genistein (20.1 min), coumestrol (20.7 min), glyceollin III (23.3 min), glyceollin II (23.6 min), and glyceollin I (23.7 min). Calibration curves with high linearity were constructed for each isoflavonoid using a series of diluted standards (daidzin and genistin were used for MGD and MGG, respectively). All HPLC analyses were run in triplicate unless otherwise stated.

Cell culture

MCF-7 cells and human embryonic kidney (HEK) 293 cells were cultured in 150-cm^2 culture flasks in DMEM supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), basic minimum essential and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin (diluted in the medium to a 1-fold concentration from either 100- or 50-fold stocks), and porcine insulin (10^{-8} mol/L; Sigma). The culture flasks were maintained in a cell incubator in a humidified atmosphere of 5% CO_2 and 95% air at 37 C. The MCF-7 cells used here (N variant) express predominantly ERα, with weak expression of ERβ, as previously described (36a).

Luciferase assays

As previously described (37, 38), MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS) for 48 h before plating. The cells were plated in 12-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. The next day the cells were transfected for 5 h in serum/supplement-free DMEM with 1 μg pGl2-ERE2X-TK-luciferase plasmid [containing two copies of the vitellogenin estrogen response element (ERE) linked to the luciferase gene; TK, tyrosine kinase] using 3 μL Lipofectamine (Life Technologies, Inc.)/μg DNA. HEK 293 cells were plated in 12-well plates at 5×10^5 cells/well in 5% CS-FBS, allowed to attach overnight, then transfected with 1 μg pGl2-ERE2X-TK-luciferase plasmid and either 500 ng pcDNA3.1B-ERα or 10 ng pcDNA3.1B-ERβ. After 5 h the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% CS-FBS containing vehicle, 17β-estradiol, phytochemical, or 17β-estradiol plus phytochemical and incubated at 37 C. After 18 h the medium was removed, and 200 μL 1 × lysis buffer (Promega Corp., Madison, WI) were added per well and

incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15,000 × *g* for 5 min. The cell extracts were normalized for protein concentration using reagent following the protocol supplied by the manufacturer (Bio-Rad Laboratories, Inc., Hercules, CA). Luciferase activity for the cell extracts were determined using luciferase substrate (Promega Corp.) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

MCF-7 cell proliferation assay

The MCF-7 cell proliferation assay used is a modified version of published methods (39–41). MCF-7 cells were placed in phenol red-free DMEM supplemented with 10% 5% CS-FBS 7 days before plating. The cells were plated in 96-well plates at 4.5×10^3 cells/well (~10% confluence) in 100 μL of the same medium. After 24 h the cells were dosed with treatment medium at 100 μL/well. Treatment medium consisted of 10% dextran-coated charcoal-FBS into which phytochemicals and controls in ethanol carrier were added (0.1% ethanol, vol/vol). The experimental cells were retreated with phytochemicals on day 4. Cell proliferation was measured on day 7 when positive control wells reached 90–100% confluence. Alamar Blue dye was added to the medium (10 μL/well), and the plates were incubated for 3 h at 37 C with 5% CO_2. Fluorescence was monitored at 560 nm excitation and 590 nm emission using a FluoroLite 1000 (Dynatech Corp., Chantilly, VA). Within proliferation assays, each dose was run in four wells. Reported data are the mean (±SD) of three independent experiments.

ERα and ERβ binding analysis

The ERα and ERβ binding assays were performed using a modification of previously reported methods (42). A PanVera CoreHTS ER kit was used for both ERα and ERβ experiments. A 26-nmol/L ERα solution was added to a fluorescent estrogen (2 nmol/L Fluormone ES2; Panvera, Madison, WI) ligand to form an ES2/ERα complex with high fluorescence polarization. Fifty microliters of the ES2/ERα complex were added to sample tubes containing 50-μL serial dilutions of test phytochemicals and were mixed well by shaking. A control tube containing 50 μL ES2 screening buffer and 50 μL ES2/ERα complex was used as a negative control to determine the polarization value with no competitor present and represented 0% competition. E_2 was used as a control on each plate. The tubes were incubated in the dark at room temperature (22 C) for 2 h. Polarization values were read using a Becon 2000 fluorescence polarization instrument (Panvera) at 485 nm excitation and 530 nm emission. Each data point in the proliferation assay was run in triplicate, and reported data are the mean (±SD) of three experiments.

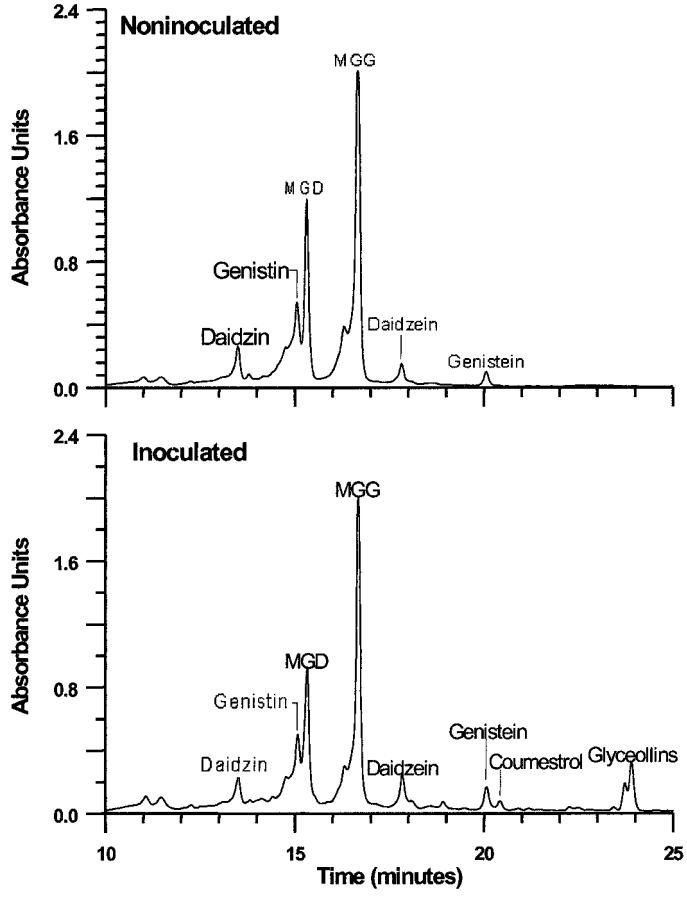

FIG. 2. Elicitor-mediated alteration of the soybean isoflavonoid profile. HPLC comparison between noninoculated and inoculated soybean cotyledons. A, HPLC chromatogram of 3-day-old noninoculated cotyledons, showing constitutive isoflavonoids; B, HPLC chromatogram of 3-day-old cotyledons inoculated with *A. sojae*, detailing the induction of coumestrol and glyceollin isomers I, II, and III. The data represent steady state amounts of glyceollins I–III and coumestrol at or near their peak levels after 3 days at 260 nm.

Results

Changes in isoflavonoid levels in cotyledons inoculated with *A. sojae* were analyzed using HPLC. A representative HPLC profile comparison between noninoculated and inoculated soybean cotyledons with *A. sojae* is displayed in Fig. 2. Figure 2A displays the HPLC chromatogram obtained from noninoculated cotyledon tissue. The more prevalent constitutive isoflavonoids, daidzin, genistin, MGD, MGG, daidzein, and genistein, are present. The HPLC assay used in this study did not detect trace levels of glyceollin in the noninoculated soybean cotyledon tissue. Figure 2B displays the HPLC chromatogram obtained from *A. sojae*-inoculated cotyledon tissue. The induction of high concentrations (1117 μg/g) of the glyceollin isomers I–III is clearly shown. This concentration of total glyceollin is relatively high compared with the concentrations of daidzein and genistein, and experiments conducted in our laboratory have indicated that glyceollin can represent up to 56% of the total isoflavonoid composition of the inoculated soybean cotyledon (see Table 1). Low levels of coumestrol were detected in inoculated soybean cotyledons (30 μg/g).

The reported estrogenic effects of soy and soy foods are primarily due to the soy isoflavonoids genistein, daidzein, and glycitein. These isoflavonoids as well as coumestrol and other flavonoids predominantly act as estrogenic chemicals, but also exhibit antiestrogenic activity in a dose-dependent manner (17, 18, 43). Therefore, both the specific type and amount of flavonoids present will determine the overall estrogenic activity. Based upon the observed differences in isoflavone profiles in normal *vs.* elicited soy, extracts were used to examine the overall estrogenic activity of soy under these two conditions. Using a estrogen-responsive reporter gene assay in MCF-7 human breast carcinoma cells we observed a difference in relative estrogenic activity between these two extracts (Fig. 3A). Although normal soy extract resulted in a maximal 94% estrogenic activity occurring at 100 μg/mL, a maximal 69% activity was observed with treated soy extract (100 μg/mL). Similar experiments were performed using elicited or normal soy extracts in combination with 17β-estradiol treatment to assess antiestrogenic activity (Fig. 3B). Normal soy extract did not exhibit antiestrogenic activity at any concentration tested, with activity in combined treatments remaining at or above that with estro-

TABLE 1. Phytoestrogen composition of noninoculated and *A. sojae*-inoculated soybean seeds

Phytoestrogen	Noninoculated soybean seed (μg/g dry wt)	*A. sojae*-inoculated soybean seed (μg/g dry wt)
Daidzein	7 ± 0.5	37 ± 10
Genistein	11 ± 1	20 ± 7
Daidzin	199 ± 40	93 ± 6
Genistin	249 ± 15	119 ± 29
Malonyldaidzin	380 ± 33	239 ± 43
Malonylgenistin	468 ± 10	344 ± 33
Coumestrol	N/D	20 ± 6
Glyceollins[a]	N/D	1117 ± 229

Results are the averages of triplicate experiments. N/D, Not detected.

[a] Total of glyceollins I, II, and III.

gen alone (100%). In contrast, the elicited soy extract decreased estrogen's activity below 100% at concentrations of 1–100 μg/mL, with a maximal decrease to 55% at 100 μg/mL. Therefore, the relative difference in isoflavone content between treated and untreated soy observed by HPLC analysis correlated with a decreased estrogenic profile in the treated soy extracts. Interestingly, this decreased estrogenic activity occurred despite increased levels of coumestrol, glyceollins I–III. Alone, coumestrol is a potent estrogenic compound, whereas glyceollins appear nonestrogenic (Fig. 4A). This suggested that the altered profile of flavonoids, particularly the presence of novel chemicals (*i.e.* glyceollins) might be responsible for the observed antiestrogenic effects of elicited soy. We next investigated the effects of isolated soy isoflavonoids on estrogenic signaling. Consistent with previous results, genistein, coumestrol, and daidzein demonstrated a dose-dependent activation of the estrogen response in MCF-7 cells (Fig. 4A), with coumestrol showing the greatest activity (90% at 100 nmol/L), followed by genistein (110% at 1 μmol/L) and daidzein (150% at 10 μmol/L). Treatment with the glyceollins from 10 nmol/L to 25 μmol/L displayed only weak activity at 10 nmol/L equivalent to 25% of that of E_2 (1 nmol/L; Fig. 4A). To determine whether the glyceollins acted as antiestrogens, MCF-7 cells were transfected with ERE-luciferase and treated with E_2 in addition to increasing concentrations of glyceollins (10 nmol/L to 25 μmol/L). As shown in Fig. 4B, these assays revealed that despite the lack of agonistic activity, the glyceollins demonstrated antagonistic activity in MCF-7 cells between 1 and 25 μmol/L concentrations. The antiestrogenic activity of glyceollins as observed in breast carcinoma cells was further evaluated using ER-positive Ishikawa human endometrial carcinoma cells. Although little or no agonistic activity was observed in these cells, glyceollins did display antagonistic activity, but at higher concentrations than observed in MCF-7 cells (data not shown).

The proliferation of MCF-7 cells is a well established biological response to 17β-estradiol and a useful screening tool for compounds that may function as estrogen agonists (38–40). Additionally, E_2-induced proliferation can be blocked by the addition of antiestrogenic compounds such as ICI 182,780 or tamoxifen. Here we demonstrate that estrogen alone is capable of stimulating MCF-7 cell proliferation (3.6 ± 1.2-fold), as measured using an Alamar Blue staining technique

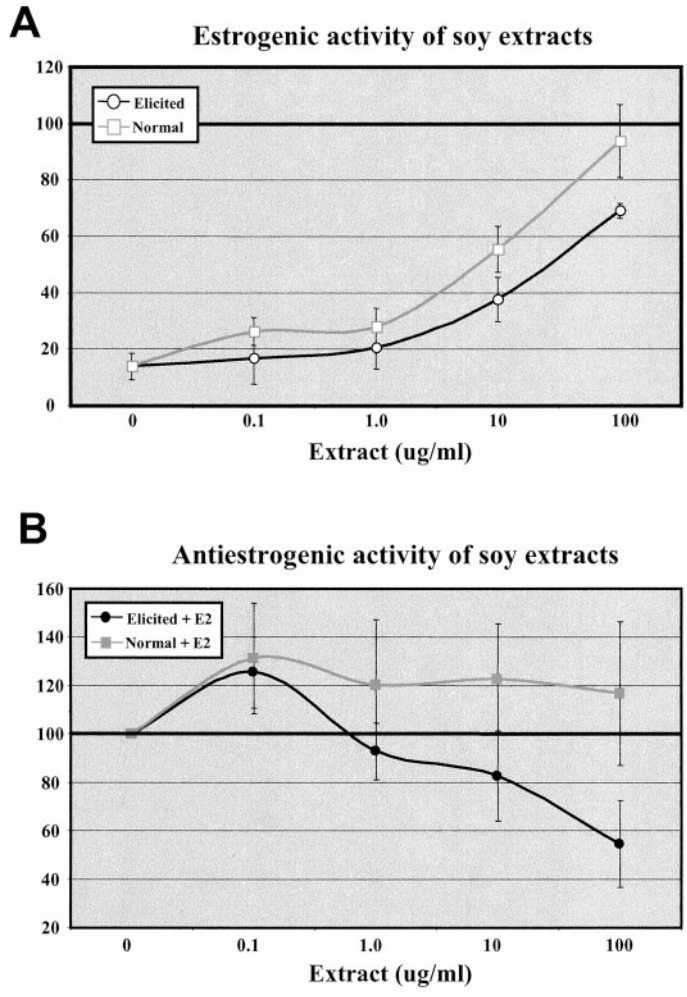

FIG. 3. Estrogenic and antiestrogenic effects of normal *vs.* elicited soy extracts. MCF-7 cells were transfected with an ERE-Luc plasmid for 6 h, treated overnight, and harvested for luciferase activity. Cells were treated with increasing doses (1–100 μg/mL) of normal (●) or elicited (●) soy extracts (A) or were treated with E_2 (1 nmol/L) in combination with normal (●) or elicited (●) soy extracts (B). Data are represented as the percent estrogenic activity as determined from 1 nmol/L E_2 alone (100%) ± SEM of three experiments.

(Fig 5). The addition of 100 nmol/L ICI 182,780 inhibited E_2-stimulated proliferation (data not shown), whereas treatment with 100 nmol/L ICI 182,780 alone maintained cell proliferation at levels similar to those after treatment with medium and carrier solvent alone (−5.2 ± 1.3%). The glyceollins alone showed a low level of estrogenic activity; however, at 10 μmol/L the estrogenic activity increased to 62%. The dose-dependent addition of the glyceollins suppressed the E_2-stimulated proliferation (100%) to 71% and 30% at 10 and 25 μmol/L, respectively. Interestingly, the glyceollins alone at 10 μmol/L were capable of increasing proliferation to 62%. However, ICI 182,780 was unable to block this proliferation, suggesting that an alternate, non-ER-related signaling pathway was involved.

There has been significant recent interest in the newly identified ERβ (25, 26). Previous studies have demonstrated some differences in ligand binding specificity and *trans*-activation between the α and β ERs (18, 26, 44, 45). Of par-

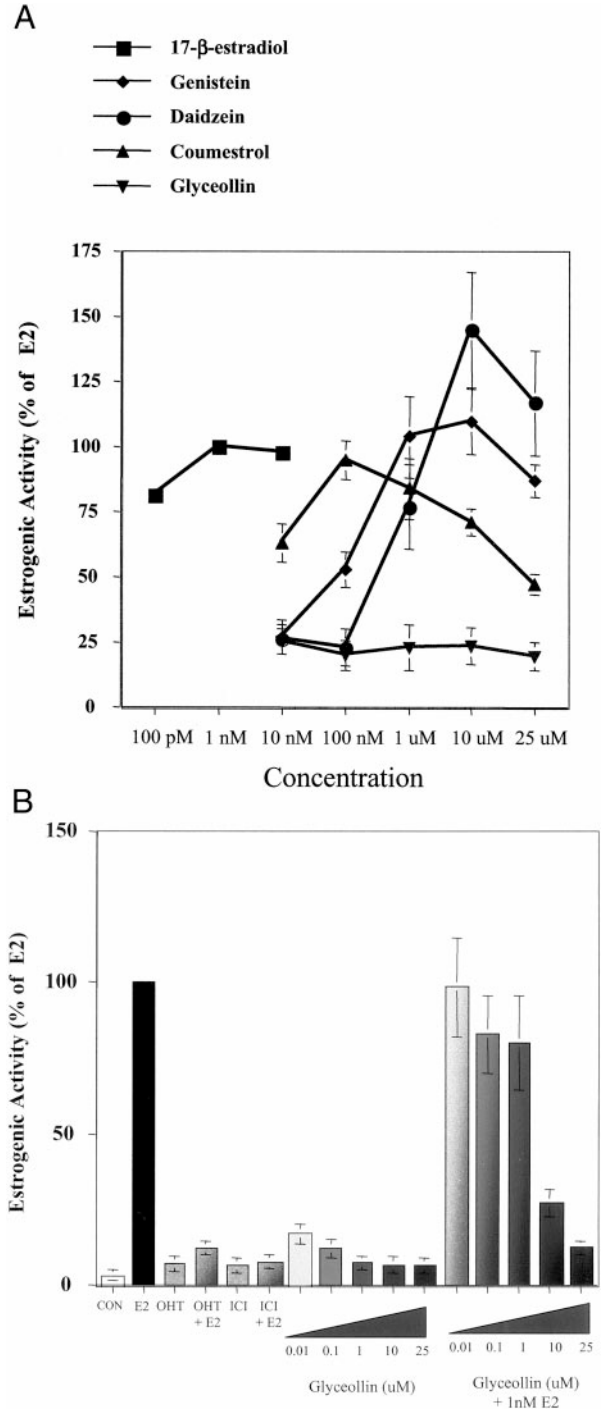

FIG. 4. Estrogenic and antiestrogenic activities of glyceollin in MCF-7 breast carcinoma cells. MCF-7 cells were transfected with an ERE-luciferase plasmid for 6 h, treated, and harvested for luciferase activity the following day. Data are presented as the percent estrogenic activity relative to 1 nmol/L E_2 (●; 100%). A, Estrogenic activity of the isoflavones daidzein (●), genistein (▲), and coumestrol (●) and the phytoalexin glyceollin (●) determined by treatment with increasing concentrations (10 nmol/L to 25 μmol/L) of phytochemical. B, Antiestrogenic activity was determined using glyceollin (10 nmol/L to 25 μmol/L) in combination with 1 nmol/L E_2. The antiestrogenic effects of glyceollin were compared with those of 100 nmol/L 4-hydroxytamoxifen (OHT) and 100 nmol/L ICI 182,780 (ICI) alone or in combination with 1 nmol/L E_2. *Data points* and *error bars* represent the mean ± SEM of three experiments per each concentration tested.

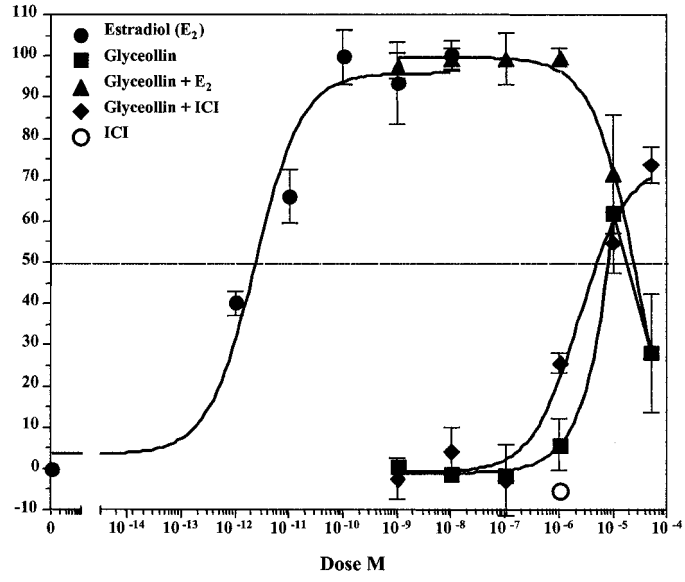

FIG. 5. Estrogenic and antiestrogenic activities of glyceollin at varying concentrations using an MCF-7 cell proliferation assay. Cell proliferation was determined using an Alamar Blue assay and is expressed relative to E_2 (100%) at 0.1 nmol/L (●). The proliferative effects of glyceollin (1 nmol/L to 50 μmol/L) are shown alone (●), in combination with 10 nmol/L E_2 (●), or in combination with 1000 nmol/L ICI 182,780 (▲). *Data points* and *error bars* represent the mean ± SD of three experiments.

ticular interest was the observation that certain flavonoid phytochemicals may bind with higher affinity and possess higher agonistic action toward ERβ (25, 26, 44–48). To assess the ability of the glyceollins to bind to ERα and ERβ, a competitive binding assay with fluorescent detection was used. Figure 6A details the results for the competitive binding assay using ERα. A displacement to 50% ES2 bound to ERα occurred at a concentration of 5 nmol/L. The IC_{50} of the glyceollins for ERα was 3.2 μmol/L. However, as shown in Fig. 6B, the IC_{50} of the glyceollins for ERβ was 6.4 μmol/L. This indicated that the ability of glyceollin to act as an ER antagonist occurred through receptor binding, and the greater affinity for ERα *vs.* ERβ correlated with the preferential antagonism of ERα activity.

To determine whether the glyceollins exhibit higher activity toward either receptor, transient transfection was performed using the ER and ER-negative cell line HEK 293. Cotransfection of either ERα or ERβ along with an ERE-luciferase construct allowed examination of the effects of receptor-specific estrogenic or antiestrogenic effects of glyceollin. Treatment with 17β-estradiol resulted in 14- and 8.4-fold *trans*-activation comparable to controls of ERα and ERβ, respectively. These results are consistent with the observations that MCF-7 cells treated with glyceollins from concentrations of 100 nmol/L to 25 μmol/L did not significantly activate an ERE response. However, ERα-transfected cells treated with 1 nmol/L E_2 at 100%, when combined with glyceollins, produced a dose-dependent decrease in ER activity to 42% and 15% at 10 and 25 μmol/L (see Fig. 7A). Using ERβ-transfected cells glyceollin was capable of suppressing β signaling to 60% and 45% of E_2 at similar glyceollin concentrations (Fig. 7B). Both the synthetic estrogen

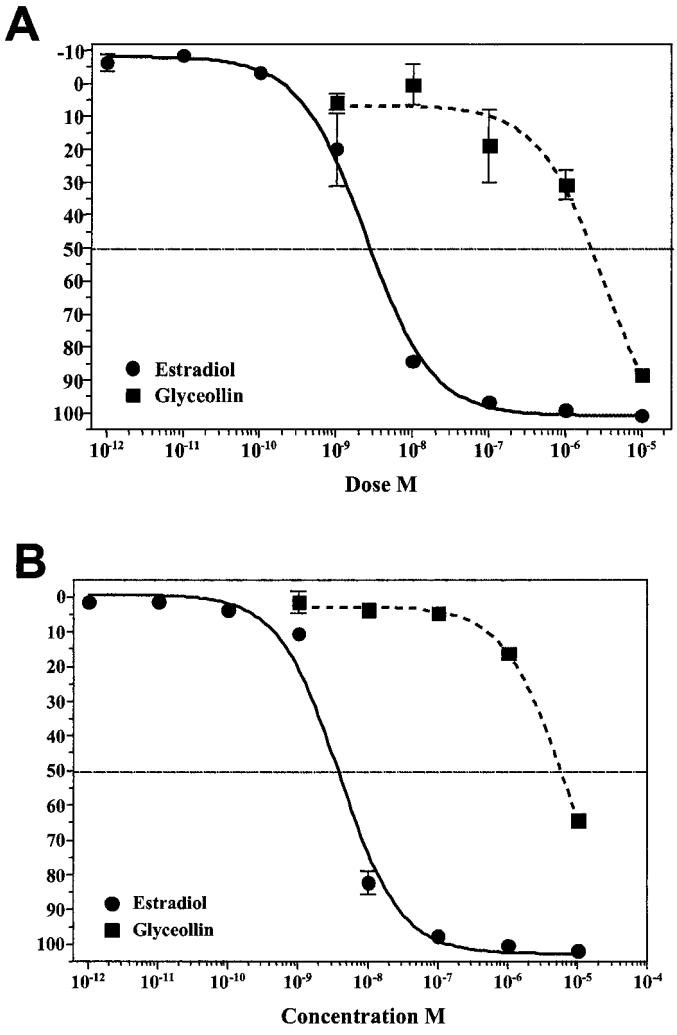

FIG. 6. Competition binding curves of glyceollin and ER (ERα and ERβ). Increasing concentrations of glyceollin (1–10 μmol/L; ●) were added to ERα/ES2 complex (A) and ERβ/ES2 complex (B) and compared with E_2 (●). *Data points* and *error bars* represent the mean ± SD of three experiments (n = 3) for each concentration tested.

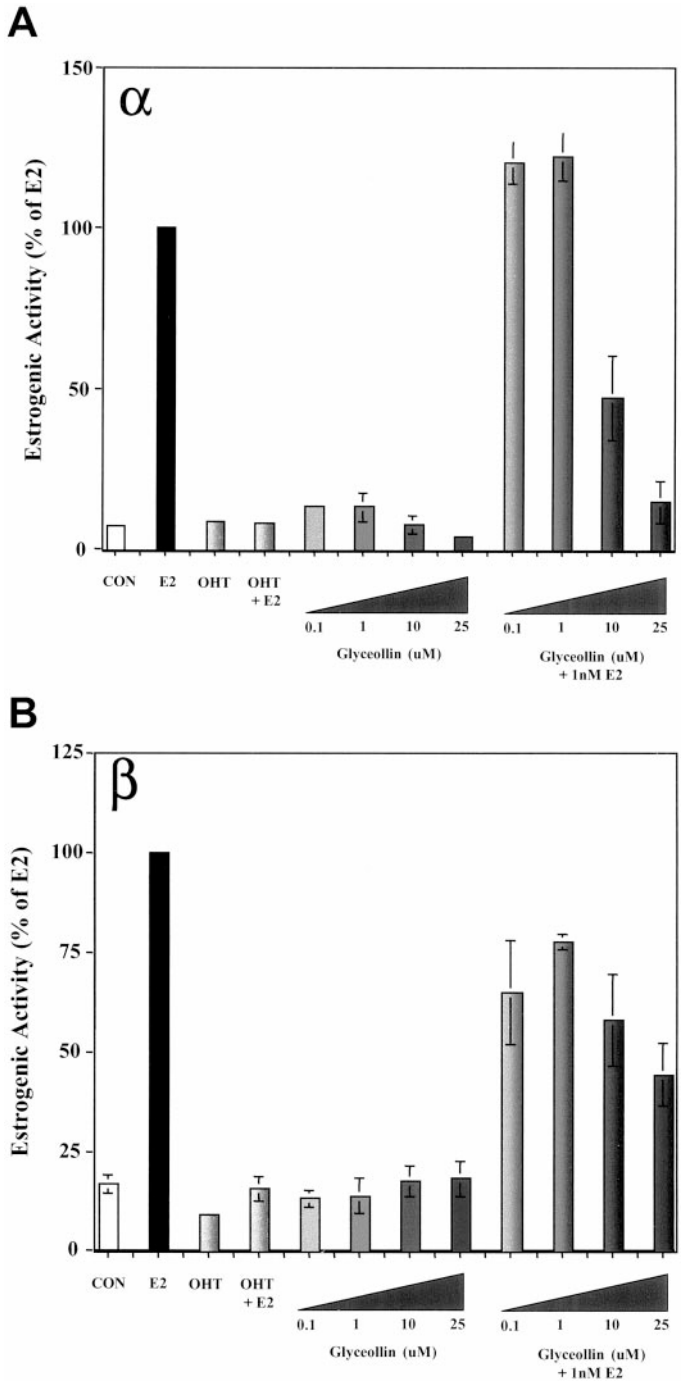

FIG. 7. ERα- and ERβ-specific effects of glyceollin. The estrogenic and antiestrogenic activities of glyceollin (0.1–25 μmol/L) were examined using HEK293 cells transfected with an ERE luciferase plasmid along with either ERα (A) or ERβ expression vectors (B), with E_2 at 1 nmol/L representing 100% activity. The antagonistic activity of glyceollin on ERα and ERβ was examined alone or in combination with 1 nmol/L E_2. *Data points* and *error bars* represent the mean ± SEM of four experiments for each concentration tested.

diethylstilbestrol (DES; 1 nmol/L) and the phytoestrogen genistein (1 μmol/L) have been shown to function as estrogens in ERα- or ERβ-transfected HEK 293 cells. Consistent with these studies, DES (1 nmol/L) and genistein (1 μmol/L) both stimulated ERE-Luc activity to a similar extent as E_2 (1 nmol/L; data not shown). The antiestrogenic effect of the glyceollins was examined using DES or genistein as an activator of ERE-luciferase. The glyceollins displayed similar preferential suppression of ERα signaling compared with ERβ activated by either DES or genistein.

Discussion

Given the significant interest in the estrogenic activity of isoflavonoids, this study was undertaken to determine the hormonal activity of the isoflavonoid phytoalexin glyceollin. Glyceollin accumulates in high concentrations in soybeans under conditions of stress, and little is know about its hormonal effects in mammalian systems. The presence of glyceollin and other phytoalexins in foods obtained from stressed plants presents a potential hazard to human health. The HPLC profiles in Fig. 2 demonstrate that glyceollin is readily extracted with daidzein and genistein and can represent as much as 56% of the total isoflavone composition. The availability of daidzein and genistein in processed soy

foods, including soy protein (49, 50), leads to the conclusion that glyceollin would be present along with the other constitutive isoflavones in soy foods prepared from treated (stressed) soy. Therefore, the glyceollins were examined in a variety of hormone-responsive systems and, in contrast to previously identified soy isoflavonoids, demonstrated antiestrogenic effects in these systems. Studies with MCF-7 cells revealed the glyceollins suppressed both E_2-mediated gene *trans*-activation and E_2-mediated proliferation when applied at similar concentrations. However, the glyceollins alone were capable of only slightly enhancing MCF-7 cell proliferation. This effect was not suppressed by combination with the antiestrogen ICI 182,780, suggesting an ER-independent mechanism. Several flavonoids have been demonstrated to influence effects on other signaling pathways, such as tyrosine kinases, mitogen-activated kinases, and protein kinase C inhibition (3, 46–48). The ability of the glyceollins to induce proliferation may therefore be mediated through an unrelated pathway. Additionally, we have shown that that certain flavonoids, unable to compete for ER binding, inhibited both E_2-mediated gene expression and proliferation, potentially through undefined alternate signaling pathways. To confirm that the antiestrogenic effects of the glyceollins occurred through direct receptor interaction, binding analyses of the glyceollins with both ERα and ERβ were performed. These studies showed that the glyceollins demonstrated a slightly greater affinity for ERα than for ERβ. The antiestrogenic activity observed in MCF-7 was further evaluated using ER-negative HEK 293 cells transfected with either ERα or ERβ. These studies demonstrated that the glyceollins suppressed E_2-induced *trans*-activation through ERα to a greater extent than ERβ. Similar results were obtained using either a known estrogenic isoflavonoid genistein or the synthetic estrogen DES. Previous reports (25, 26, 44, 45) demonstrated greater binding to and activation of ERβ *vs.* ERα by phytoestrogens. In contrast to these reports, the antiestrogenic effects of the glyceollins appear to be due to the greater affinity toward ERα.

Significant research has previously identified a potential role for soy and soy foods in the prevention of human disease and the promotion of health. These effects, including decreased risk of certain types of cancers as well as prevention of cardiovascular disease and osteoporosis, have been linked to the estrogenic isoflavonoids genistein and daidzein present in soy. However, the relative amounts of these two isoflavonoids and the glucose-conjugated forms vary dramatically among soybean varieties (28, 30) and the type of soy food prepared (49, 50). Additionally, daidzein and genistein are not the only isoflavonoids found in soybeans. The recent report by Song *et al.* demonstrated the estrogenic activity of glycitein, an isoflavonoid also detected in both soy and soy foods (27). Extensive work has shown that the amount and type of isoflavonoids found within legumes are dependent upon plant growth conditions, and that biosynthesis of these compounds can be significantly altered under conditions of stress (4, 28, 29, 31–36). The type and amount of these compounds may influence the overall estrogenic activity of soy-based foods. We have demonstrated both estrogenic and antiestrogenic effects of numerous other flavonoid compounds (17, 18), suggesting that isoflavonoids besides genistein and daidzein may be important in the health benefits of these compounds. Recent studies have also demonstrated that flavonoids from red clover and hops (23, 51) possess estrogenic effects and may represent important considerations in human health. Here we describe the phytochemical isoflavonoid glyceollin as being induced in soybean plants grown under conditions of stress. The lack of agonistic activity of the glyceollins in combination with weak, but significant, antiestrogenic activity are of interest. In contrast to the observed estrogenic effects of many soy isoflavonoids and other flavonoids, the antiestrogenic effects of glyceollins may also be considered important with regard to their presence in soy-based foods.

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Erratum

In the article "Degree of fatness after treatment for acute lymphoblastic leukemia in childhood" by Karsten Nysom, Kirsten Holm, Kim Fleischer Michaelsen, Henrik Hertz, Jørn Müller, and Christian Mølgaard (*The Journal of Clinical Endocrinology & Metabolism* **84:**4591–4596), Table 1 was incorrectly reproduced. Under the heading "whole body percent fat," in the line entitled "mean z-score (95% CI)," the footnote symbols are incorrect. Reading left to right the symbols *a*, *c*, *a*,*c*, and *a*,*c* should read *a*, *b*,*c*, *a*,*b*, and *a*,*c*. Footnote *c* should be labeled *b*,*c*. The printer regrets the error.