3-24-1999

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Estrogenic Activity of Glycitein, a Soy Isoflavone

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Glycitein (4',7-dihydroxy-6-methoxyisoflavone) accounts for 5–10% of the total isoflavones in soy food products. The biological activity of this compound has not been reported to date, although numerous studies have been performed with the other soy isoflavones, daidzein and genistein. Glycitein was isolated from soy germ to 99% purity. Weaning female B6D2F1 mice were dosed with glycitein (3 mg/day), genistein (3 mg/day), and diethylstilbestrol (DES) (0.03 μg/day) in 5% Tween 80 by gavage for 4 days. A control group received an equal volume of 5% Tween 80 solution daily. The uterine weight increased 150% with glycitein (p < 0.001), 50% with genistein (p < 0.001), and 60% with DES (p < 0.001) compared with the control group. DES, 17β-estradiol, and three isoflavones (daidzein, genistein, and glycitein) were examined for their competitive binding abilities with 17β-(3H)estradiol to the estrogen receptor proteins of the B6D2F1 mouse uterine cytosol. The concentrations of each compound required to displace 50% of the (3H)estradiol at 5 nM in the competitive binding assay were 1.15 nM DES, 1.09 nM 17β-estradiol, 0.22 μM genistein, 4.00 μM daidzein, and 3.94 μM glycitein. These data indicated that glycitein has weak estrogenic activity, comparable to that of the other soy isoflavones but much lower than that of DES and 17β-estradiol.

**INTRODUCTION**

Estrogens play important hormonal roles among all vertebrates. Animal estrogens are exclusively steroidal compounds, and the principal physiological estrogen in most species is 17β-estradiol. Many plants produce compounds that possess estrogenic activity in animals and are, thus, called phytoestrogens. These compounds have some structural similarity to the mammalian estrogen, 17β-estradiol. A common structural characteristic of many of these compounds is the presence of a phenolic ring, a prerequisite for the binding to the estrogen receptor (Figure 1).

Soybeans contain the highest concentrations of isoflavones, at 1–3 mg/g, as daidzein, genistein, glycitein, and their corresponding glucosides, of foods consumed by humans. These soy isoflavones may have some important health-enhancing properties such as prevention of certain cancers (Barnes et al., 1991), lowering the risk of cardiovascular diseases (Anderson et al., 1995), and improvement of bone health (Bahram et al., 1996). Their estrogenic activities may play an important role in their health-enhancing properties. Genistein and daidzein account for the major portion of isoflavones in soy foods and have been the focus of numerous studies. The estrogenic activities of daidzein, genistein, and their glucoside forms, genistin and daidzin, were 100 000–500 000 times lower than that of diethylstilbestrol (DES) as assessed by the mouse uterotropic assay (Farmakalidis and Murphy, 1985). Estrogen receptor binding studies with soy isoflavones, genistin and daidzin, demonstrated they had the abilities to bind to estrogen receptors from different species including mice, rats, and sheep (Verdeal et al., 1980). No biological studies on glycitein have been reported to date. However, glycitein accounts for 5–10% of the total isoflavones in soy foods and may be as high as 40% in some soy supplements composed of soy germ (Song et al., 1998; Tsukamoto et al., 1995). Therefore, it is important to evaluate the biological activity of glycitein. Because glycitein has a structure similar to those of genistein and daidzein, we hypothesize that it will have estrogenic activity.

There are a number of methods to assess estrogenic activity. Reel et al. (1996) proposed a test array for potential estrogenic activity. These methods can be grouped into four categories: (1) estrogen receptor binding; (2) estrogen receptor-dependent transcriptional expression; (3) reproductive tract response; and (4) nonreproductive tract target tissue response. It is preferable to perform more than one assay to confirm the results. The binding affinities of soybean isoflavones were much lower than those of estradiol and DES (Verdeal et al., 1980). Farmakalidis and Murphy (1985) evaluated the estrogenic activity of genistin and daidzin, the isoflavone glucosides, and showed that the glucosides had estrogenic activity equal to that of the aglycons on a molar basis. The mouse uterine enlargement assay has been the standard in vivo method to evaluate estrogenic activity (Bickoff et al., 1962), and, because it is performed in an intact animal, the effects of absorption, metabolism, serum binding, and pharmacokinetics are taken into account. These two methods were used to establish the estrogenic activity of glycitein.

**MATERIALS AND METHODS**

**Chemicals.** DES, Tween 80, 17β-(2,4-3H)estradiol (23 mCi/mmol), 17β-estradiol, and dextran-coated charcoal (DCC) were obtained from Sigma Chemical Co. (St. Louis, MO). ACS

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scintillation fluid was purchased from Fisher Scientific Inc. Glycitein was purified according to the following method: 10 g of soy germ (generously donated by Schouten USA, Inc., Minneapolis, MN) was hydrolyzed in 100 mL of 0.1 N HCl at 98 °C for 2 h, evaporated at 50 °C, and filtered through a Whatman No. 42 filter paper. The filtrate was dried by a rotary evaporator at 50 °C. The residue was dissolved in 80% ethanol at 98 °C for 2 h, extracted with acetonitrile, and filtered through a Sephadex LH-20 column (2.5 cm × 50 cm) with 50% ethanol as the eluent. The glycitein peak was collected and freeze-dried. Genistein was purified from soybeans using the same procedure as glycitein. Daidzein was chemically synthesized according to the method of Song et al. (1998). The identification and purity of glycitein, daidzein, and genistein were confirmed by HPLC, ultraviolet (UV) spectral analysis, melting point, and mass spectrum analysis. Glycitein, daidzein, and genistein were analyzed by HPLC on a Beckman System Gold chromatography system consisting of a model 507 autosampler, a model 126 dual pump, a model 168 photodiode array detector, and an IBM 486 computer with Beckman Gold system HPLC data processing software (version 8, 1993) according to the procedure of Murphy et al. (1997). A YMC-pack ODS-AM-303 column (5 μm, 25 cm × 4.6 mm) (YMC Inc., Wilmington, NC) was used. UV spectral analysis was performed according to the method of Mabry et al. (1970) using a Beckman DU 7400 spectrophotometer. The melting point was measured with a Perkin-Elmer 7 series differential scanning calorimeter (DSC) (Perkin-Elmer Inc., Norwalk, CT). Mass spectrum analysis using direct probe introduction and chemical ionization was performed on a Finnigan model TSQ-700 mass spectrometer (Finnigan Inc., Piscataway, NJ).

Figure 1. Chemical structures of soy isoflavones, 17β-estradiol, and DES.

Animals and Treatments. The mice uterine enlargement assay was performed according to the method of Farmakalidis and Murphy (1985). Inbred B6D2F1 mice were obtained from Harlan Sprague–Dawley (Madison, WI). Each dam was housed separately with her female pups (14 days old on arrival), and the mice were used. DCC (2.5%, 0.5 mL) in 1 mM EDTA buffer was added to the mixture, mixed well, and centrifuged at 10000 × g for 10 min. After centrifugation, the supernatants were decanted into scintillation vials containing 10 mL of ACS scintillation fluid. The samples were counted for 10 min on a Packard liquid scintillation analyzer model 1900TR (Packard Instrument Co., Downers Grove, IL).

Plasma Isoflavone Analysis. Plasma samples of the genistein group and the glycitein group were combined to give one pooled sample for each treatment. Two milliliters of 0.2 M sodium acetate, pH 5.5, buffer, 100 μL of β-glucuronidase/ sulfatase (Sigma Chemical Co.), and 20 μL of 4 mM internal standard, 2,4,4′-trihydroxydeoxybenzoin (THB) (Song et al., 1998), were added to 2 mL of plasma sample. The mixture was centrifuged at 37 °C for 16 h. Six milliliters of methanol was added to the mixture, mixed well, and centrifuged at 10000 × g for 20 min. Eight milliliters of supernatant was brought to dryness and dissolved in 400 μL of 80% methanol. After centrifugation, 20 μL of sample was taken for isoflavone analysis by our HPLC system. The HPLC conditions were the same as in the identification of genistein and glycitein stated above. The minimal detection level by UV detector at 254 nm for genistein and glycitein was 0.5 μg/mL or 1.9 μM in injection solution.

Statistical Analysis. The uterine enlargement data were analyzed by using a one-way classification analysis of a completely randomized design (SAS version 6, SAS Institute Inc., Cary, NC) at α = 0.05.
RESULTS AND DISCUSSION

The purity of glycitein and genistein was confirmed by HPLC, UV spectral analysis, melting point, and mass spectrum analysis. The HPLC chromatography of glycitein showed one single peak. The purity of glycitein was confirmed by peak area percentage and peak purity program to be >99%. Naim et al. (1973) first isolated glycitein from soybeans and reported a melting point of 337 °C. The purity of genistein was confirmed to be 99% by using the similar method for glycitein.

The uterine enlargement assay yielded significant differences (p < 0.001) in uterine weights among treatments (Table 1). The glycitein, genistein, and DES groups had 150, 50, and 60% greater uterine weight, respectively, compared with the control. The relative estrogenic potencies of DES, genistein, and glycitein were estimated on the basis of the doses of estrogen required to produce a 10-mg increase of uterine weight. The estrogenic potencies of DES, genistein, and glycitein were 100000, 1, and 3, respectively (Table 1). The glycitein, genistein, and DES were 100000, 1, and 3, respectively (Table 1). The relative estrogenic potencies of DES, genistein, and glycitein were estimated on the basis of the doses of estrogen required to produce the same increase of uterine weights.

The relative estrogenic potencies of genistein and DES from the present study were similar to the results of Farmakalidis and Murphy (1985), who found the relative molar potencies of DES, genistein, genistin, and daidzein to be 100000, 1, 1, and 0.26, respectively. The present study demonstrated that the estrogenic potency of glycitein in mice uterine enlargement assay was 3 times higher than that of genistein, but, as hypothesized, was much lower than that of DES.

It is generally considered that nonsteroidal estrogens exert their stimulatory effect on the estrogen receptor by binding to the same site as that occupied by steroidal estrogens such as 17β-estradiol. Our competitive binding study results are shown in Figure 2, in which increasing concentrations of unlabeled estrogens displace bound (3H)estradiol. These results confirm that glycitein has the ability to bind to the estrogen receptor. DES had a much higher binding affinity compared with that of the three isoflavones. The relative affinities of these compounds for the mice estrogen receptor were calculated by dividing the CB50 of unlabeled 17β-estradiol by the CB50 of a competitor and then multiplying by 100 (Table 2). These data, once again, confirmed that both DES and estradiol had a much greater binding affinity to estrogen receptor than the three isoflavones. Among the three soybean isoflavones, genistein had the greatest estrogen receptor binding affinity. The in vitro estrogen receptor binding data in this study were comparable to the previous results summarized by Verdeal et al. (1980). They reported that genistein had a binding affinity roughly 100 times lower than that of 17β-estradiol, and daidzein had a binding affinity 10–15 times lower than that of genistein.

The plasma extraction and analysis recovery was 85% for internal standard THB. The recovery-corrected plasma isoflavone concentrations for genistein-treated and glycitein-treated group pooled sample were 0.42 and 0.72 µM, respectively. Xu et al. (1994) and King et al. (1995) have reported in humans and in rats that 4–6 h after isoflavone dosing, daidzein and genistein reached maximal concentrations in plasma and then decreased thereafter. At 24 h after dosing, there were only trace concentrations of daidzein and genistein in the plasma. Because we collected blood 24 h after the last dose of isoflavones, the low plasma isoflavone concentrations may be the result of rapid metabolism or excretion of isoflavones after dosing.

Table 1. Estrogenic Activities of Soy Isoflavones in Mice

<table>
<thead>
<tr>
<th>treatment</th>
<th>total dose</th>
<th>no. of mice</th>
<th>uterine wt (mg, mean ± SEM)</th>
<th>body wt (g)</th>
<th>relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0 mg</td>
<td>20</td>
<td>17.7 ± 0.9</td>
<td>8.1</td>
<td>11.6</td>
</tr>
<tr>
<td>DES</td>
<td>12 mg</td>
<td>20</td>
<td>28.5 ± 3.1</td>
<td>8.1</td>
<td>11.7</td>
</tr>
<tr>
<td>genistein</td>
<td>12 mg</td>
<td>20</td>
<td>44.6 ± 3.1</td>
<td>8.3</td>
<td>12.1</td>
</tr>
</tbody>
</table>

a Uterine weight means bearing different letters are significantly different, p < 0.05. b The relative potency was calculated on the basis of the doses of estrogen required to produce a 10-mg increase of uterine weight.

Table 2. Relative Affinities of Estrogens for Estrogen Receptors

<table>
<thead>
<tr>
<th>compound</th>
<th>CB50 (nM)</th>
<th>relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>0.00109</td>
<td>100</td>
</tr>
<tr>
<td>DES</td>
<td>0.00115</td>
<td>95</td>
</tr>
<tr>
<td>genistein</td>
<td>0.223</td>
<td>0.49</td>
</tr>
<tr>
<td>daidzein</td>
<td>4.00</td>
<td>0.027</td>
</tr>
<tr>
<td>glycitein</td>
<td>3.94</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* Based on the molar concentrations (CB50) required to displace 50% of the (3H) estradiol. n = 2.
Glycitein gave a much lower in vitro binding affinity compared to genistein in the estrogen receptor-binding assay. However, it gave higher estrogenic response in the in vivo mouse uterine enlargement assay. This may be the result of a higher bioavailability of glycitein compared to that of genistein in mice. In a human isoflavone metabolic study, a higher bioavailability of glycitein compared with that of genistein was demonstrated (Zhang et al., 1999). Shutt et al. (1970) reported that genistein could be metabolized to p-ethylphenol, which is not an estrogenic compound. It may be possible that glycitein is metabolized to compounds with greater estrogenic potency than that of genistein.

Daidzein has been reported to be a less effective estrogen than genistein when fed to mice (Bickoff et al., 1962). Our present study demonstrated that glycitein is a stronger estrogen in mice uterine enlargement assay compared to genistein. Of the three soy isoflavone aglycons, it appears that glycitein has the highest estrogenic potency in the in vivo estrogen assay.

This study has demonstrated the in vivo estrogenic activity and the in vitro binding affinity to estrogen receptors of glycitein. These data reveal that, although glycitein accounts for ~10% of the total isoflavones in soy foods, its biological potency is comparable to those of the other soy isoflavones. Development of soybean and soy food isoflavone databases needs to include glycitein and its glucosides. Additionally, the biological activity of glycitein and its glucosides in humans needs to be explored.

LITERATURE CITED


Zhang, Y.; Wang, G. J.; Song, T. T.; Murphy, P. A.; Hendrich, S. Glycitein is a more bioavailable soybean isoflavone than is daidzin in humans having moderate fecal isoflavone degradation activity. J. Nutr. 1999, in press.

Received for review September 21, 1998. Revised manuscript received February 18, 1999. Accepted February 19, 1999. This work was supported by the U.S. Army Breast Cancer Research Initiative and Material Command, Grant DAMD 17-MM4529WVM, the Center for Designing Foods To Improve Nutrition, Iowa State University, and the Iowa Agriculture & Home Economics Experiment Station and published as J. Nutr. Paper J-17287, Project 3353.