Isoflavones modulate the glucuronidation of estradiol in human liver microsomes

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Soy food has been associated with a reduced incidence of hormonal cancer in Asian countries, and the soy isoflavones daidzein and genistein are believed to protect against tumors induced by the endogenous hormone 17β-estradiol (E2). In the present study, we have examined if daidzein and genistein as well as several structurally related isoflavones are able to modulate the in vitro glucuronidation of E2 in human hepatic microsomes. It is known that different isoforms of UDP-glucuronosyltransferase (UGT) are involved in E2 glucuronidation: UGT1A1 leads exclusively to the 3-glucuronide and is stimulated by E2 via homotropic kinetics, whereas UGT2B7 gives rise to the 17-glucuronide of E2 following Michaelis–Menten kinetics. In our study, daidzein markedly stimulated the 3-glucuronidation, thereby enhancing the metabolic clearance of E2. In contrast, genistein inhibited the 3-glucuronidation. The 17-glucuronidation of E2 was not affected by either compound. Formononetin and the daidzein metabolites equol, 3′-hydroxy-daidzein, 6-hydroxy-daidzein and glycitein behaved similar to daidzein, whereas biochanin A resembled genistein. The effect of daidzein on the 3-glucuronidation of E2 in human hepatic microsomes was also obtained with human recombinant UGT1A1. Since the only other compound known to stimulate E2 glucuronidation via allosteric kinetics is 17α-ethynylestradiol, our study is the first report of the heterotropic stimulation of a UGT by a non-steroidal and naturally occurring compound. An enhanced rate of glucuronidation of E2 by daidzein and its metabolites may contribute to the putative protection of soy against hormonal cancer.

Introduction

The endogenous steroid hormone 17β-estradiol (E2) is believed to play a major role in the etiology of breast and endometrial cancer (1,2). Although the mechanisms of E2-mediated carcinogenesis are still a matter of debate, there is a general consensus that cancer risk increases when plasma and tissue levels of E2 are elevated over prolonged periods of time (3,4). The incidence of breast cancer is ~4-fold lower in Far East Asian nations, such as Japan as compared with the Western World, e.g. the USA, Canada and Western Europe (5,6). One frequently offered explanation for this difference is the high consumption of soy food in Asia (7). Soy-containing foods are an abundant source of isoflavone phytoestrogens, and research over the past decade suggests that these compounds may protect against hormone-dependent cancers (8–12). Various mechanisms have been proposed for the beneficial effects of soy isoflavones: in addition to an antiestrogenic activity exerted by the weak estrogenic isoflavones in the presence of E2, inhibitory effects of soy isoflavones on enzymes involved in the biosynthesis of E2, e.g. aromatase and 17β-hydroxysteroid dehydrogenase, have been reported (13). Such inhibition of E2 biosynthesis would lower the plasma and tissue levels of free E2, as would stimulation of the synthesis of the transport protein sex hormone binding globulin (12). However, a decrease of E2 concentrations in plasma and tissues might also be achieved if the disposition of E2 could be stimulated. The major pathway of E2 elimination comprises its conjugation with glucuronic acid in the liver and excretion of the conjugate into the bile and also into the urine after transport to the kidneys (14).

Owing to its two hydroxyl groups, E2 is able to form two different glucuronides (Figure 1), which are known to involve different isoforms of UDP-glucuronosyltransferases (UGTs) and exert different biological activities. The glucuronide at position 3 of E2 (E2-3-G) is known to be formed in humans by several UGTs, e.g. UGT1A8 in the intestine; however, E2-3-G is specifically formed in human liver by UGT1A1, an enzyme previously known as bilirubin UGT and also catalyzing the glucuronidation of numerous xenobiotics (15). In 2000, Fisher et al. (16) reported that UGT1A1 undergoes homotropic activation by E2; this finding became possible after accessibility of the membrane-bound UGT was increased by treatment with the pore-forming peptide alamethicin.

![Fig. 1. The two forms of E2-3- and -17-glucuronide.](https://academic.oup.com/carcin/article/26/12/2172/2390803)

Abbreviations: E2, 17β-estradiol; E2-3-G, 17β-estradiol-3-glucuronide; E2-17-G, 17β-estradiol-17-glucuronide; HLM, human liver microsomes; HPLC, high performance liquid chromatography; UGT, uridine diphosphoglucuronosyltransferase.
The atypical enzyme kinetic is described by the Hill equation. In contrast, the formation of 17β-estradiol-17-glucuronide (E2-17-G) in humans is preferentially catalyzed by UGT2B7 with minor contributions of UGT1A3, 1A4 and 2B15, and follows the classical Michaelis–Menten kinetics (17). The type of kinetics is important for the estimation of the intrinsic clearance, because the metabolic clearance is overestimated if a Michaelis–Menten kinetics is erroneously assumed instead of a Hill kinetics (18). For E2, only the formation of the 3-glucuronide represents an inactivating pathway, because the 17-glucuronide is biologically active and known to inhibit the bile flow, thereby causing cholestasis (19).

The aim of the present study was to elucidate the effect of various isoflavone phytoestrogens present in soy-based food, i.e. daidzein, genistein and glycitein, as well as related isoflavones and metabolites of daidzein (Figure 2) on the glucuronidation of E2 in microsomes from human liver. Pronounced effects were observed for some but not all isoflavones on the 3-glucuronidation of E2, which should result in faster elimination of E2 and may thus have a bearing on E2-mediated carcinogenesis.

Materials and methods

Chemicals and materials

E2, E2-3-G, E2-17-G, the isoflavones depicted in Figure 2 (with the exception of equol), and all other biochemicals and reagents were from Sigma/Aldrich/Fluka (Taufkirchen, Germany). Equol was purchased from Indofine (Hillsborough, FL). Human liver obtained from a 63-year-old male white individual (Hillsborough, FL). Human liver obtained from a 63-year-old male white individual (Hillsborough, FL). Human liver obtained from a 63-year-old male white individual (Hillsborough, FL). Human liver obtained from a 63-year-old male white individual (Hillsborough, FL). Supersomes, i.e. microsomes from insect Sf-9 cells infected with baculovirus strain containing human UGT1A1 cDNA, were from Gentest (Woburn, MA). β-Glucuronidase/arylsulfatase from Helix pomatia was purchased from Roche Diagnostic (Mannheim, Germany).

Preparation of microsomes

Human liver microsomes (HLM) were prepared from the human liver according to the method of Lake (20). Protein concentration was determined by the method of Bradford (21) using BSA as standard.

Glucuronidation assay

Microsomes from HLM or supersomes containing human UGT1A1 were used in modified enzymatic assays according to the methods of Fisher et al. (16) and Matern et al. (22). In a typical incubation, 0.1 mg microsomal or supersonical protein and 25 μg amethicin were mixed in ~50 μl of 0.1 M potassium phosphate buffer pH 7.1 and placed on ice for 15 min. MgCl₂ (final concentration 10 mM), the β-glucuronidase inhibitor saccharolactone (5 mM) and the substrates E2 and/or isoflavone dissolved in dimethylsulfoxide (DMSO) (final DMSO concentration was 1%) were added, and the mixture was preincubated at 37°C for 5 min. To initiate the reaction, UDPGA (final concentration 4 mM) was added to give a final volume of 200 μl and further incubated for 30 min. Product formation was linear with time for at least 60 min and 120 min for E2-3-G and E2-17-G, respectively. The assays were terminated by adding 200 μl of 0.7 M glycine–HCl buffer pH 1.2. In an aliquot of 50 μl, protein was sedimented by centrifugation and the free isoflavone and its glucuronides were directly determined by high performance liquid chromatography (HPLC). The remaining 350 μl of the incubation mixture were extracted with 2 × 800 μl ethylacetate, the extract was evaporated to dryness, the residue dissolved in methanol and analyzed by HPLC to measure E2 and its glucuronides. This work-up of the incubation mixture was necessary because pilot studies had shown that only the isoflavones and their glucuronides are completely dissolved in the aqueous supernatant after centrifugation, whereas a part of the E2 was adsorbed to the microsomes (E.Pfeiffer, unpublished data). A complete extraction from the incubation mixture with ethylacetate was observed for E2, both E2-glucuronides and the isoflavones but not the isoflavone glucuronides.

Quantification of E2 and its glucuronides as well as the unconjugated isoflavones was achieved by calibration with the reference compounds. The limit of detection, defined as 3-fold baseline noise, was 25 pmol for E2-3-G, 12 pmol for E2 and E2-17-G, and 1–5 pmol for the isoflavones. For comparison, the amounts of E2-3-G and E2-17-G measured by HPLC in incubations of the lowest concentration of E2 (5 μM) were 100–150 and 50–100 pmol, respectively.

For the identification of the isoflavone glucuronides, each isoflavone was incubated with HLM for 30 min as described above but without saccharolactone. An aliquot of the incubation mixture after protein sedimentation was analyzed by HPLC using gradient I (see below). Another aliquot was mixed with the same volume of 0.15 M acetate buffer pH 5.0 and 10 μl of a β-glucuronidase/arylsulfatase preparation from Helix pomatia, and incubated at 37°C for 16 h. After protein sedimentation, the supernatant was analyzed by HPLC using gradient I. The peaks present in the chromatogram of the non-hydrolyzed sample but absent after enzymatic hydrolysis were assumed to represent glucuronides. Since the sum of the peak areas before and after hydrolysis was identical, the same molar extinction coefficient was used for the quantification of the isoflavone glucuronides and the unconjugated isoflavones.

Fig. 2. Chemical structures of isoflavones studied for their effects on E2 glucuronidation.

Daidzein stimulates E2 glucuronidation
isoﬂavones. Each glucuronidation experiment comprised three parallel incubations, i.e. E2 alone, the isoflavone alone and E2 together with the isoflavone.

**HPLC analysis**

A Beckman System Gold HPLC with a binary HPLC pump, a photodiode array detector, and a Nouveau Software for data collection and handling was used. Separation was carried out on a reversed-phase Phenomenex Luna C8(2) column (250 mm × 4.6 mm i.d., particle size 5 µm). Eluents were A distilled water adjusted to pH 3.0 with formic acid and B acetonitrile, with a flow rate of 1 mL/min. Two different linear gradients were employed: gradient I started with 17% B for 2 min, then changed to 45% B over 7 min, then to 50% B over 7 min, then to 100% B over 5 min, followed by 5 min at 100% B; gradient II changed from 20% B to 28% B during the ﬁrst 27 min, to 50% B over 7 min, hold for 2 min at 50% B, then to 60% B over 3 min and ﬁnally at 100% B for 5 min.

**Enzyme kinetic analysis**

Visual inspection of ﬁtted functions (velocity as a function of substrate concentration) and Edie–Hofstee plots (velocity as a function of velocity/substrate concentration) was used to select the best-ﬁt enzymic kinetic model (18). This include the Michaelis–Menten model \( V = \frac{V_{\text{max}} \times S}{K_m + S} \) and the substrate activation model \( V = V_{\text{max}} \times S^n(K_m + S)^{n-1} \), where \( n \) is the Hill coefﬁcient or the degree of sigmoidicity of the curve. For the Michaelis–Menten model, the estimated values of intrinsic clearance \( CL_{\text{int}} \) were calculated based on the equation \( CL_{\text{int}} = \frac{V_{\text{max}}}{K_m} \) whereas for the sigmoid model the maximal clearance \( CL_{\text{max}} \) was calculated using the equation \( CL_{\text{max}} = \frac{(V_{\text{max}}/K_m)^{1/n} \times (n-1)(n \times (n-1)^{1/n})}{1/n} \) (18).

**Results**

When E2 was incubated with HLM in the presence of UDPGA, the formation of two glucuronides was observed by HPLC analysis, which were identiﬁed as E2-3-G and E2-17-G by cochromatography with reference compounds. The retention times obtained with two different HPLC gradients are listed in Table I. The kinetics of the glucuronide formation was then determined and it was shown that 3-glucuronidation exhibited homotropic activation kinetics consistent with the Hill equation, whereas 17-glucuronidation followed Michaelis–Menten kinetics. The kinetic values obtained from three independent experiments over a concentration range of 5–100 µM E2 were \( V_{\text{max}} = 975 \pm 25.4 \) pmol/min/mg protein, \( K_{0.5} = 21.0 \pm 3.7 \) µM and the Hill coefﬁcient \( n = 1.7 \pm 0.20 \) for the 3-glucuronidation, whereas the 17-glucuronidation had \( V_{\text{max}} = 184 \pm 17.7 \) pmol/min/mg protein and \( K_m = 11 \pm 3.2 \) µM.

It was of interest to compare the glucuronidation activity of our HLM with data published in the literature. HLM obtained from human donors of diverse age and either gender are commercially available from Gentest (Woburn, MA); the reported average activity of 15 commercial HLM at 100 µM E2 was

<table>
<thead>
<tr>
<th>Isoﬂavone</th>
<th>2174</th>
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</thead>
</table>

**Table I.** HPLC retention times (min) of the unconjugated E2 and isoﬂavones and their glucuronides with two different HPLC gradients

<table>
<thead>
<tr>
<th>HPLC gradient I</th>
<th>E2-3-G</th>
<th>E2-17-G</th>
<th>E2</th>
<th>Isoﬂavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>12.7</td>
<td>13.5</td>
<td>17.2</td>
<td>12.6</td>
</tr>
<tr>
<td>G2</td>
<td>9.7</td>
<td>11.2</td>
<td>12.8</td>
<td>10.1</td>
</tr>
<tr>
<td>G3</td>
<td>6.6</td>
<td>10.9</td>
<td>12.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>16.2</td>
<td>n.a.</td>
<td>21.3</td>
<td>32.0</td>
</tr>
<tr>
<td>Glycitein</td>
<td>13.5</td>
<td>n.a.</td>
<td>16.4</td>
<td>29.8</td>
</tr>
<tr>
<td>Equol</td>
<td>10.8</td>
<td>12.6</td>
<td>15.0</td>
<td>19.0</td>
</tr>
<tr>
<td>6-HO-daidzein</td>
<td>6.8</td>
<td>8.4</td>
<td>9.2</td>
<td>11.0</td>
</tr>
<tr>
<td>3'-HO-daidzein</td>
<td>7.7</td>
<td>10.2</td>
<td>10.9</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Table II: HPLC retention times (min) of the unconjugated E2 and isoﬂavones and their glucuronides with two different HPLC gradients

<table>
<thead>
<tr>
<th>HPLC gradient I</th>
<th>E2-3-G</th>
<th>E2-17-G</th>
<th>E2</th>
<th>Isoﬂavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>31.7</td>
<td>33.0</td>
<td>38.4</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>9.7</td>
<td>11.2</td>
<td>12.8</td>
<td>10.1</td>
</tr>
<tr>
<td>G3</td>
<td>6.6</td>
<td>10.9</td>
<td>12.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>16.2</td>
<td>n.a.</td>
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<td>32.0</td>
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<td>Glycitein</td>
<td>13.5</td>
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</tr>
<tr>
<td>Equol</td>
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</tr>
<tr>
<td>6-HO-daidzein</td>
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<td>9.2</td>
<td>11.0</td>
</tr>
<tr>
<td>3'-HO-daidzein</td>
<td>7.7</td>
<td>10.2</td>
<td>10.9</td>
<td>11.8</td>
</tr>
</tbody>
</table>

G1, G2, G3, different glucuronides formed from the same isoﬂavone; n.a., not applicable because this isoﬂavone can form only one glucuronide.

598 (range 180–1025) pmol/min/mg protein for 3-glucuronidation and 186 (range 75–387) pmol/min/mg protein for 17-glucuronidation. When the glucuronidation activities at 100 µM E2 were determined with our HLM, values of 940 and 162 pmol/min/mg protein were determined for 3- and 17-glucuronidation, respectively, which are in good agreement with the Gentest data. The ratio of E2-3-G to E2-17-G formed under these conditions was reported as 3.7 (range 1.2–10.8) for the Gentest HLM and determined as 5.8 for our HLM, conﬁrming that our HLM exhibit the typical E2-glucuronidation activity of human hepatic microsomes.

The isoﬂavones daidzein, genistein, glycitein, biochanin A, formononetin as well as three metabolites of daidzein, i.e. 6-hydroxy-daidzein, 3'-hydroxy-daidzein and equol (for chemical structures see Figure 2) were examined for their modulatory effect on the glucuronidation of E2 using our HLM as the enzyme source. Since a single HPLC gradient was not sufﬁcient to separate E2 and its glucuronides from all the isoﬂavones and their glucuronides, the more suitable of two different HPLC gradients was selected for each isoﬂavone (Table I). Gradient I was used for biochanin A, formononetin, 6-hydroxy-daidzein and 3'-hydroxy-daidzein, whereas Gradient II was used for daidzein, genistein, glycitein and equol.

In the absence of isoﬂavone, 25 µM E2, which is near the substrate concentration for half-maximal velocity, was converted to an extent of 32% to E2-3-G with an activity of 549 ± 44.0 pmol/min/mg microsomal protein, and to an extent of 8% to E2-17-G with the rate of 136 ± 9.2 pmol/min/mg microsomal protein (mean of 24 experiments). The formation of E2-3-G and E2-17-G from 25 µM E2 in the presence of 25 µM of various isoﬂavones was then determined and expressed as a percentage of the respective glucuronidation of E2 in the absence of isoﬂavone (Figure 3). The two major soy isoﬂavones daidzein and genistein exhibited markedly different effects; whereas daidzein stimulated 3-glucuronidation of E2 by ~50% and inhibited 17-glucuronidation by ~15%, genistein gave rise to a strong inhibition of the formation of E2-3-G but did not affect E2-17-glucuronidation (Figure 3). Thus, daidzein shifted the 3- and 17-glucuronidation of E2 into different directions, although the stimulating effect predominated. Glycitein, formononetin and the daidzein metabolites 6-hydroxy-daidzein, 3'-hydroxy-daidzein and equol behaved like daidzein, although the effects were less pronounced, whereas biochanin A had the same effect as genistein (Figure 3).

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Incubation of the various isoflavones with HLM in the presence of UDPGA and subsequent analysis by HPLC showed that all isoflavones were good substrates of the UGTs present in HLM: conversion to glucuronides ranged from 65 to 100%, and up to three different glucuronides were formed, depending on the number of hydroxyl groups of the respective isoflavone (Table II). The exact structures of the glucuronides could not be determined owing to the lack of reference compounds, but the glucuronide nature was clearly demonstrated by the UDPGA-dependent formation and by subsequent enzymatic hydrolysis to yield the unconjugated isoflavone. The extent of glucuronidation of isoflavones was ~3-fold higher than observed for E2. When the glucuronidation of the isoflavones was determined in the presence of an equimolar concentration of E2, a reduction of 20–30% was observed (Table II). When microsomes from insect Sf-9 cells expressing human UGT1A1 were used for the glucuronidation of E2, only E2-3-G but no E2-17-G was formed. Daidzein, genistein and glycitein exhibited the same effect on the formation of E2-3-G by recombinant UGT1A1 as observed with HLM, i.e. a pronounced stimulation by daidzein and glycitein but a marked inhibition by genistein (Figure 4). In contrast to E2, the glucuronidation of the three isoflavones by the recombinant UGT1A1 gave rise to the same pattern of glucuronides as was obtained with HLM (data not shown).

The stimulating effect of daidzein on the 3-glucuronidation, but not 17-glucuronidation, of E2 was clearly demonstrated in further experiments with HLM. When various concentrations of E2 ranging from 5 to 200 μM were glucuronidated in the absence and in the presence of 25 μM daidzein, more of the 3-glucuronide was formed in the presence of the isoflavone (Figure 5A1). The stimulation was a moderate 10% at 200 μM E2 but reached almost 100% at 5 μM E2, where daidzein had a 5-fold molar excess over E2. When the velocity V of the 3-glucuronidation of E2 in the absence and presence of 25 μM daidzein and the E2 concentrations were plotted according to Eadie–Hofstee, a typical hooked curve consistent with homotropic activation kinetics was observed in the absence of daidzein; however, this curve shifted to a more linear relationship consistent with Michaelis–Menten kinetics in the presence of daidzein (Figure 5A2). The metabolic clearance (CL) of E2-3-G, calculated from these data, was elevated from 24 μl/min/mg protein in the absence of daidzein to 62 μl/min/mg protein in the presence of 25 μM daidzein.

### Table II. Glucuronidation of isoflavones in human liver microsomes in the absence and presence of E2

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Glucuronides number (ratio)</th>
<th>Conversion (% of dose)</th>
<th>Glucuronidation activity (pmol/min/mg protein)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>without E2</td>
<td>with E2</td>
</tr>
<tr>
<td>Daidzein</td>
<td>2 (90/10)</td>
<td>78</td>
<td>1259 ± 42.1</td>
<td>838 ± 30.9</td>
</tr>
<tr>
<td>Genistein</td>
<td>2 (93/7)</td>
<td>82</td>
<td>1364 ± 60.7</td>
<td>1034 ± 19.7</td>
</tr>
<tr>
<td>Glycitein</td>
<td>2 (99.5/0.5)</td>
<td>100</td>
<td>&gt;1600</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>1</td>
<td>65</td>
<td>1104 ± 28.3</td>
<td>879 ± 20.1</td>
</tr>
<tr>
<td>Formononetin</td>
<td>1</td>
<td>91</td>
<td>1453 ± 32.1</td>
<td>1169 ± 13.6</td>
</tr>
<tr>
<td>3'-HO-daidzein</td>
<td>3 (90/7/3)</td>
<td>80</td>
<td>1289 ± 35.8</td>
<td>815 ± 14.2</td>
</tr>
<tr>
<td>3'-HO-daidzein</td>
<td>3 (47/21/32)</td>
<td>81</td>
<td>1329 ± 20.2</td>
<td>857 ± 61.0</td>
</tr>
<tr>
<td>Equol</td>
<td>2 (78/22)</td>
<td>94</td>
<td>1577 ± 3.5</td>
<td>1402 ± 23.3</td>
</tr>
</tbody>
</table>

Concentration of the isoflavone and E2 was 25 μM. The values given for the ratio and conversion rate were obtained in the absence of E2. Data represent the mean ± standard deviation of three independent experiments.
In another experiment, a fixed concentration of 25 \( \mu \text{M} \) ME 2 was used and the effects of varying concentrations of daidzein on the 3- and 17-glucuronidation of E2 were examined (Figure 5B1). Enhanced formation of E2-3-G was observed between 5 and 50 \( \mu \text{M} \) daidzein, whereas higher concentrations of daidzein inhibited the 3-glucuronidation of E2. When 3-glucuronidation was enhanced, 17-glucuronidation was decreased, probably as a result of the decreased availability of E2 as substrate for 17-glucuronidation.

When the velocity and substrate concentration of the glucuronidation of daidzein in the absence and presence of E2 were plotted according to Eadie–Hofstee, enzyme kinetics according to Michaelis–Menten were obtained (Figure 5B2). \( V_{\text{max}} \) and \( K_m \) were 2220 pmol/min/mg protein and 25 \( \mu \text{M} \), respectively, in the absence of E2, and 2230 pmol/min/mg protein and 59 \( \mu \text{M} \), respectively, in the presence of E2. Thus, E2 inhibited the glucuronidation of daidzein in a competitive manner.

**Discussion**

Breast cancer risk is associated with elevated tissue and plasma levels of ovarian hormones, in particular E2 (3,4). E2 regulates breast cell proliferation and can therefore promote breast cancer cell growth. Moreover, oxidative metabolism of E2 in the liver and in estrogen target tissues, such as breast and uterus, leads to estrone (E1) and to various hydroxylation products, some of which, i.e. 16\( \alpha \)-hydroxy-E1 and the 4-hydroxylated catechol metabolites of E2 and E1, exhibit genotoxic activity and may contribute to E2-mediated carcinogenesis (23,24). E2 and its oxidative metabolites can only be efficiently excreted following glucuronidation, because sulfate conjugates have been shown to release the steroidal estrogens again after hydrolysis by intracellular sulfatases (25).

Glucuronidation of ovarian estrogens is also important because it abolishes their biological activity (25). Thus, the activities of UGT enzymes involved in the glucuronidation of E2 and its metabolites are key determinants for the homeostasis of steroidal estrogens.

In human liver, E2 is glucuronidated at two positions of the molecule. The formation of E2-17-G follows Michaelis–Menten kinetics and appears to be catalyzed by several UGTs. In contrast, E2-3-G is a specific product of UGT1A1, and the kinetics of its formation are consistent with homotropic activation, meaning that E2 can stimulate its own glucuronidation at position 3 (16). In a recent study using HLM, the synthetic estrogen 17\( \alpha \)-ethynylestradiol, but none of eight flavonoids, was found to stimulate the 3-glucuronidation of
E2 at low concentrations, thus representing the first report of a heterotropic activation of UGT1A1 (26).

The results of the present study clearly demonstrate that several isoflavones also have the ability to stimulate the 3-glucuronidation of E2 in HLM (Figure 3). The most active compound was daidzein, which is one of the two major isoflavones present in soybeans and many soy-based food items. The daidzein metabolites equol, glycitein, 6-hydroxy-daidzein and 3'-hydroxy-daidzein were also heterotropic activators of E2-3-glucuronidation, whereas the other major soy isoflavone, genistein, did not stimulate but inhibited the formation of E2-3-G (Figure 3). From the limited number of isoflavones in our study (Figure 2) it can be concluded that the presence of a hydroxy group at position 5 of the isoflavone molecule, e.g. in genistein and biochanin A, abolishes the activating effect, whereas hydroxy or methoxy groups at other positions of the A and B rings have little or no influence (Figure 3). The effect of daidzein is specific for 3-glucuronidation, because the formation of E2-17-G by HLM was not stimulated. The amount of E2-17-G appears to solely depend on the concentration of E2 available for 17-glucuronidation, because it was slightly decreased when 3-glucuronidation was enhanced and increased when 3-glucuronidation was inhibited (Figure 5B1).

The differential effects of daidzein, genistein and glycitein on microsomes from transgenic insect cells expressing solely UGT1A1 were the same as observed with HLM (Figure 4), confirming that UGT1A1 is the target of the modulating effect of the isoflavones in human liver.

When hepatic microsomes from male and female Sprague–Dawley were used under the same experimental conditions as HLM, 3-glucuronidation of E2 was not stimulated (E.Pfeiffer, unpublished data), although it is known that rat and human UGT1A1 are highly homologous and have very similar substrate specificity (27). 17-Glucuronidation of E2 was markedly preferred over 3-glucuronidation in our study with rat liver microsomes and has previously also been observed by Alkharfy and Frye (28). The higher metabolic efficiency for 17-glucuronidation over 3-glucuronidation may explain the failure to detect any effect of daidzein. Thus, the rat may not be a good animal model for the glucuronidation of E2.

The stimulation of 3-glucuronidation of E2 by daidzein and some of its metabolites as well as by 17α-ethynylestradiol represents the first example for the heterotropic activation of a conjugating enzyme. However, this phenomenon is well established for several cytochrome P450 (CYP) isoforms, in particular human CYP3A4, which is important for the oxidation of numerous endogenous and exogenous compounds. Several of these oxidations follow autoactivation kinetics and are heterotropically stimulated by certain drugs (reviewed in 29). Most of these activations have been demonstrated in microsomal systems and only very few in vivo. For example, the formation of diclofenac metabolites by CYP3A4 was stimulated by quinidine in human liver microsomes; similar results were obtained with monkey liver microsomes, and the diclofenac clearance was significantly enhanced by quinidine in monkeys in vivo (30,31). The kinetic data from in vitro studies are highly important for predicting the in vivo clearance of compounds (18).

If the heterotropic activation of 3-glucuronidation of E2 by daidzein and its metabolites takes place in vivo, it may have a bearing on the homeostasis of E2 in liver and other tissues, because the excretion of E2 should be expedited resulting in lower serum and tissue levels. A 30–80% decrease in the serum levels of E2 on various days of the menstrual cycle of premenopausal women kept on a soy diet for 1 month have been reported (32). However, the study design does not allow to conclude whether this was because of the heterotropic activation of UGT1A1 or the induction of this or other UGTs. No studies have been published to date on the effect of isoflavones on tissue levels of E2. Of particular interest would be target tissues for E2 carcinogenicity, e.g. the breast and endometrium, both of which express UGT1A1 (25,33). Moreover, the catechol estrogens 2-hydroxy-E2 and 2-hydroxy-E1, as well as their methylation products are also substrates of UGT1A1 but follow Michaelis–Menten kinetics with much higher metabolic efficiency than observed for the formation of E2-3-G (33). For the glucuronidation of 4-hydroxy-E2, 4-hydroxy-E1 and their methylation products, UGT1A1 appears to be of low importance as these metabolites are efficiently conjugated by UGT2B7 (33).

Soy contains about equal amounts of daidzein and genistein. Since genistein has an inhibiting effect on the 3-glucuronidation of E2 in vitro, it may be assumed that the effect of daidzein is antagonized if soy is consumed. However, the tissue levels of daidzein and genistein in humans after ingestion of soy are not known to date. Studies in rats and humans have shown that the parent isoflavones and their glucuronides and sulfates are the major products excreted after ingestion of soy or the individual isoflavones, but small amounts of reductive and oxidative metabolites are also formed (34,35). Although the phase I metabolites of daidzein also stimulate the glucuronidation of E2, the effects of the respective genistein metabolites are still unknown.

The differential effects of daidzein and genistein on the activity of human UGT1A1 reported here are another example for the notion that isoflavones with highly similar chemical structures may exhibit quite different biological activities. For example, it has been recently demonstrated that genistein has genotoxic potential, inducing micronuclei containing acentric chromosomal fragments in various mammalian cell systems in vitro and structural chromosomal aberrations in cultured human lymphocytes, whereas daidzein is devoid of such genotoxicity (34,36). Furthermore, genistein stimulates the growth of breast cancer cells inoculated into athymic mice (37), whereas daidzein does not (Helferich, personal communication). A recent study in rats showed that daidzein improves the capacity of tamoxifen to prevent mammary tumors induced by 7,12-dimethylbenzanthracene, but genistein has an opposing effect (38).

In conclusion, we have shown that daidzein and several of its metabolites cause the heterotropic activation of the 3-glucuronidation of E2, mediated by UGT1A1, in human liver microsomes. Daidzein may, therefore, lead to lower levels of E2 in these human tissues depending on UGT1A1 for the glucuronidation of E2. It is proposed to investigate whether this effect of daidzein contributes to the protection against E2-mediated cancer attributed to isoflavone-containing soy diets. Although the liver is the major site for the phases I and II metabolism of E2, the glucuronidation of E2 and its modulation by isoflavones should now also be studied in target tissues for the carcinogenic effect, e.g. the breast and uterus, using appropriate cell lines and more physiological concentrations of E2 and isoflavones. Moreover, it should be considered to use daidzein alone instead of the soy isoflavone mixture in studies designed to disclose the protective effect of isoflavones, and eventually in nutritional supplements.
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References


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