Lack of an Inducible Effect of Dietary Soy Isoflavones on the mRNA Abundance of Hepatic Cytochrome P-450 Isozymes in Rats

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Modulation of the activity and content of cytochrome P-450 (CYP) in hepatic microsomes may be important to human health since these enzymes activate and inactivate a wide range of xenobiotics and food components. Regulation of the inducibility of most CYPs involves transcriptional regulation and post-transcriptional mRNA stabilization. We examined in the present study the effect of dietary soy isoflavone (0–300 mg of isoflavone/kg of diet) on the mRNA abundance of rat hepatic CYP1A1, CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A1, CYP3A2 and CYP4A1 by quantitative competitive RT-PCR and real-time monitored RT-PCR. A fermented soy extract containing 155 mg/g of genistein, 127 mg/g of daidzein, and other minor isoflavones was used as the isoflavone source. The dietary soy isoflavone had no affect on the hepatic mRNA abundance of these CYPs. The results by both methods were well matched and indicate that the dietary soy isoflavone did not cause the induction of CYPs by transcriptional step-up regulation or post-transcriptional mRNA stabilization.

Key words: soy isoflavone; mRNA abundance; cytochrome P450; rat

Epidemiological and experimental researchers have provided extensive information on the beneficial effects of soy isoflavones on human health.1–4) A possible explanation for some of these effects, such as a preventive effect on mammary tumors, is the anti-estrogenic effect of soy isoflavones.2) Several researchers have reported that the estrogenic effect of soy isoflavone was responsible for the prevention of osteoporosis which had been caused by an estrogen deficiency in postmenopausal women or ovariectomized animals.5–6) Isoflavones have scavenging activity and anti-oxidative properties and can inhibit lipoprotein oxidation in serum and LDL oxidation, as well as exerting an atherogenic effect.7) Isoflavones are widely used as functional foods.

Cytochrome P-450 (CYP) comprises a group of drug-metabolizing enzymes involved in the detoxification of xenobiotics and in the synthesis of cholesterol, steroids and such other important lipids as prostacyclins and thromboxane A2 in the liver.8) Modulation of the activity and content of CYP in liver microsomes may be important to human health, since these enzymes activate and inactivate a wide range of xenobiotics and food components. It has been well established that dietary constituents modulated the activity and content of CYP in liver microsomes.9) The metabolism of foreign chemicals by CYP does not always lead to detoxification of these compounds. In some cases, the oxidized or reduced products have been shown to initiate chemical carcinogenesis, mutagenesis, drug toxicity and teratogenicity.10) Flavonoids, including the isoflavones just described, have had beneficial effects related to their anti-oxidative activity, but some flavonoids affected the activity and/or content of CYP, enhanced the activation of carcinogens and/or influenced the metabolism of drugs via the induction of CYP.11) Information about the effect of isoflavones on CYP is important but very limited.

The CYP enzymes are classified into families and subfamilies on the basis of their sequence similarity. Approximately 1,000 CYPs have been identified. The CYP pool varies in a species-specific manner. Isozymes in most subfamilies may be strongly induced after being pretreated with an appropriate xenobiotic. Regulation of the inducibility of most CYPs involves transcriptional regulation and post-transcriptional mRNA stabilization.12–14) The profile of the mRNA abundance of CYPs therefore provides important information. We examine in the present study the effect of dietary soy isoflavone on the gene expression of major CYPs.
Methods and Materials

This study was approved by the Laboratory Animal Care Committee of Ehime University, and the rats were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

Soy isoflavones. A fermented soybean extract (FSBE) rich in the two major isoflavone aglycones, genistein and daidzein (Kikkoman, Soy Act, Noda, Japan), was used as the source of the isoflavone aglycones. This FSBE contained 155 mg/g of genistein, 127 mg/g of daidzein, 18 mg/g of glycitein and less than 1 mg/g of genistin, daidzin and glycytin.

Animals and diets.

Experiment 1. Male Sprague Dawley rats of about 300 g BW were used. The rats were acclimatized by feeding on a commercial solid diet (MF/C212; Oriental Yeast Co., Osaka, Japan) ad libitum for 7 d. The rats were housed in individual cages with screen bottoms of stainless steel in a room maintained at 23 ± 1°C with a 12 h light-dark cycle (light, 0700–1900 h). After their acclimatization, the rats were assigned to groups of 2 rats. The each rats were given free access to either the control diet, a diet containing 300 mg of isoflavone/kg of diet or a diet containing 2,500 mg of indole-3-carbinol (I3C)/kg of diet (Table 1) for 4 wk. I3C was used as a positive control of CYP induction. Horn et al. have reported that I3C significantly induced CYP 1A1 and 3A1 in a dose-dependent manner.15) The rats were sacrificed by decapitation at midnight in a non-fasted state on the last day of the experimental period. The liver was immediately removed after scaringification, frozen in liquid nitrogen and stored at −80°C until needed for analysis.

Experiment 2. Five-week-old male and female breeder Sprague Dawley rats (Nippon SLC, Shizuoka, Japan) were fed and acclimatized as described for experiment 1. After their acclimatization, the male and female rats were assigned to two groups of 6 rats each having approximately the same body weight. The rats were given free access to either the control diet (C diet) or to a diet containing 100, 200 or 300 mg of isoflavone/kg of diet (the I100, I200 or I300 diet) and water for 4 wk. The body weight and level of food intake by weight were recorded daily for each rat in the morning before replenishing the diet. The rats were sacrificed and sampled as described for experiment 1.

Isolation of the mRNA from the liver. Total RNA was isolated from the liver according to the method described by Chomczynski and Sacchi.16) The RNA was dissolved in 50 μl of milli Q water. The concentration of RNA was measured from the absorbance at 260 nm, the ratio at 260/280 being between 1.6 and 1.9. The total RNA solution was diluted to 150 μl of total RNA/150 μl by DEPC-treated water, and then 150 μl of 20 mM Tris buffer at pH 7.5 containing 2 mM EDTA, 0.2% SDS and 15 μl of Oligotex-dT30 (Takara Bio, Shiga, Japan) was added. The mixture was allowed to stand for 3 min at 70°C, incubated for 10 min at room temperature and then centrifuged at 20,000 x g for 30 sec at room temperature. The resulting precipitate was washed 2 times with 350 μl of a 10 mM Tris buffer

Table 1. Composition and Isoflavone Content of the Control Diet, Isoflavone-added Diet and Indole-3-carbinol-added Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>I-100</th>
<th>I-200</th>
<th>I-300</th>
<th>I3C</th>
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<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Corn oil⁶</td>
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<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
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<td>Vitamin mixture⁸</td>
<td>10</td>
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<td>10</td>
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<tr>
<td>Carbohydrate</td>
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<td></td>
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<tr>
<td>Sucrose⁹</td>
<td>350</td>
<td>349.835</td>
<td>349.665</td>
<td>349.5</td>
<td>348.75</td>
</tr>
<tr>
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<td>349.835</td>
<td>349.665</td>
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</tr>
<tr>
<td>Soyact¹¹</td>
<td>—</td>
<td>0.33</td>
<td>0.67</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>Indole-3-carbinol¹²</td>
<td>—</td>
<td>—</td>
<td>mg/kg</td>
<td>—</td>
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<tr>
<td>Isoflavone content</td>
<td>—</td>
<td>100</td>
<td>200</td>
<td>300</td>
<td>—</td>
</tr>
</tbody>
</table>

¹ I-100, control diet supplemented with FSBE as 100 mg of isoflavone/kg of diet.
² I-200, control diet supplemented with FSBE as 100 mg of isoflavone/kg of diet.
³ I-300, control diet supplemented with FSBE as 100 mg of isoflavone/kg of diet.
⁴ I3C, control diet supplemented with 2.5 g of indole 3-carbinol/kg of diet.
⁵ Purchased from New Zealand Dairy Board, Wellington, New Zealand.
⁶ Purchased from Nisshin Oil Mills, Tokyo, Japan.
⁷ Based on AIN-76.
⁸ The AIN-76 vitamin mixture used in this study contained 20 g of choline bitartrate/100 g.
⁹ Granulated beet sugar, purchased from Nippon Beet Sugar Manufacturing, Tokyo, Japan.
¹⁰ Purchased from Nihon Shokuhin Kako, Tokyo, Japan.
¹¹ Purchased from Kikkoman Co., Ltd., Chiba, Japan.
¹² Purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.
at pH 7.5 containing 1 mM EDTA, 0.1% SDS and 0.1 M NaCl, before eluting the mRNA 3 times with DEPC-treated water at 70°C so that the elute was in aliquots of 50 µl. Washing and elution were conducted with a spin-column set (Centricron UFC30HV00, 0.45 µm pore size, Nippon Millipore, Tokyo Japan) at 20,000 x g for 30 sec at room temperature. The mRNA solution was stored at −80°C until needed for cDNA generation.

**Determination of the mRNA abundance of the CYPs by quantitative competitive RT-PCR.** Quantitative competitive RT-PCR for CYP1A1, CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A1, CYP3A2 and CYP4A1 was conducted with a commercial competitive RT-PCR kit (Rat Cytochrome P450 Competitive RT-PCR Set, Takara Bio, Shiga, Japan) and RT-PCR kit (Takara RNA LA PCR Kit (AMV) Ver. 1.1, Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. The sequences of the primers are not disclosed by the manufacturer for patent reasons. The product size of each target gene was as follows: CYP1A1, 332; CYP1A2, 237; CYP2B1/2, 550; CYP2C11, 249; CYP2E1, 474; CYP3A1, 581; CYP3A2, 117; CYP4A1, 345; and cyclophilin, 266. The product size of each competitor RNA was as follows: CYP1A1, 264; CYP1A2, 266; CYP2B1/2, 438; CYP2C11, 300; CYP2E1, 378; CYP3A1, 456; CYP3A2, 141; CYP4A1, 275; and cyclophilin, 320. After denaturing the mRNA for 5 min at 70°C, cDNA was synthesized at 42°C for 1 h in a final volume of 100 µl by using 5 µl of an oligo-dT primer (2.5 pmol/µl), 1 µl of total RNA (500 ng/µl), 5 µl of rat CYP RNA competitor corresponding to each isozyme (1 x 10^7, 4 x 10^7, 1.6 x 10^8 or 6.4 x 10^8 copies/µl), 5 µl of reverse transcriptase (XL AMV RTase, 5 U/µl), 50 µl of a 10 mM dNTP mixture, 10 µl of a 10x RNA PCR buffer, 25 mM of MgCl_2, 41.5 µl of DEPC-treated water and 2.5 µl of an RNase inhibitor (40 U/µl). Due to the high abundance of the mRNA of CYP2C11, the diluted mRNA (25 ng/µl) was applied to reverse transcription. The amplification reaction was conducted with 50 µl of a mixture containing 4 µl of 10x LA PCR buffer II Mg^{2+}-free, 1 µl of a 10 pmol primer set corresponding to each isozyme, 3 µl of 25 mM MgCl_2, 0.25 µl of Taqpolymerase (5 U/µl) and 31.75 µl of DEPC-treated water. After 2 min of heating at 94°C, 24 cycles were performed of 0.5 min denaturation at 94°C, 0.5 min annealing at 56°C, and 0.5 min extension at 72°C. The reaction products (3 µl) were electrophoresed on 3% agarose gel (NuSieve 3:1, Cambrex Corporation, NJ, USA) in a Tris borate/EDTA buffer and visualized by staining with 1 µg/ml of ethidium bromide. The density of the band of the target gene products was compared with those of competitor RNAs grossly or densitometrically by using the Image Beta 4.02 densitometric analysis program for Windows (Scion Corporation, MD, USA), the copy number of competitor RNA whose band showed the most compatible density with that of the target gene being regarded as the copy number of the target gene.

**Determination of the gene expression of CYPs by the real-time-monitored polymerase chain reaction method.** After denaturing the mRNA for 5 min at 70°C, cDNA was synthesized at 42°C for 1 h in a final volume of 100 µl by using 5 µl of the oligo-dT primers (500 ng/ml), 1 µl of mRNA, 5 µl of reverse transcriptase (XL (AMV) for RT-PCR, 5 U/µl, Takara Bio, Shiga, Japan), 10 µl of a 10 mM dNTP mix (PCR Nucleotide Mix, Roche, Tokyo, Japan), 10 µl of a 100 mM Tris-HCl buffer, and 2.5 µl of RNase (from human placenta, 40 U/µl, Takara Bio, Shiga, Japan). Primer sets for CYP1A1, CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A1, CYP3A2 and CYP4A1 contained in the commercial determination kit (Rat Cytochrome P450 Competitive RT-PCR Set, Takara Bio, Shiga, Japan) and real-time monitored PCR kit (LightCycler fast-start DNA master SYBR-Green I, Roche Diagnostic Co., Tokyo, Japan) were used after optimizing the Mg concentration. Briefly, 2 µl of hepatic cDNA from the rats fed on the control diet (for all CYPs except CYP1A1) or I3C-containing diet (for CYP1A1) were analyzed by real-time RT-PCR in a final reaction buffer volume of 20 µl containing SYBR-Green I dye, 2, 3, 4 or 5 mM MgCl_2, and 0.5 µM of the 5’ and 3’ primers. Each reaction mixture was transferred to a glass capillary in the LightCycler instrument according to the manufacturer’s instructions, using fluorescence detection for SYBR-Green I with an excitation wavelength of 470 nm and an emission wavelength of 530 nm. After 10 min activation of the polymerase at 95°C, PCR proceeded with denaturation for 10 s at 95°C, annealing for 10 s at 66°C and extension for 20 s at 72°C. After amplification, melting consisted of one cycle at 95°C for 5 s, 45°C for 10 s, and then increasing the temperature to 90°C at a rate of 0.1°C/s. Based on the peak sharpness and simplicity of the derivative melting curves [−d (fluorescence)/dT vs. temperature], the PCR products were tested for purity. Agarose gel electrophoresis was performed to confirm the product size and absence of primer dimmer or polymer. All primers did not formed primer dimer and showed a single sharp peak on the melting curve at 5 mM of Mg concentration (Fig. 1A and B). The PCR products were extracted from the band corresponding to the molecular weight by centrifuge-membrane filtration (Freeze’n Squeeze spin column, Nihon Bio-Rad Laboratories, Tokyo, Japan). The PCR products were diluted stepwise, analyzed by real-time-monitored PCR under the optimized conditions just described, and the real-time PCR efficiency were calculated from the given slopes by the LightCycler software (Fig. 1C). The corresponding real-time PCR efficiency (E) for one cycle in the exponential phase was calculated according to equation 1.17

\[ E = 10^{(1-slope)} \]
The hepatic cDNA samples were analyzed by real-time-monitored PCR under the optimized conditions and linearity range already described, and the relative gene expression was calculated by equation 2, using the crossing point (CP) of each target gene of CYPs and cyclophilin as the reference gene and the corresponding real-time PCR efficiency ($E_{\text{target}}$ and $E_{\text{ref}}$).

\[
\text{Relative gene expression} = \frac{E_{\text{target}}(\text{CP}_{\text{sample}}/C_0 - \text{CP}_{\text{control}})}{E_{\text{ref}}(\text{CP}_{\text{sample}}/C_0 - \text{CP}_{\text{control}})}
\]

Table 2 and Fig. 2 shows the hepatic mRNA

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>CYP1A1</th>
<th>CYP1A2</th>
<th>CYP2B1/2</th>
<th>CYP2C11</th>
<th>CYP2E1</th>
<th>CYP3A1</th>
<th>CYP3A2</th>
<th>CYP4A1</th>
<th>Cyclophilin</th>
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<tbody>
<tr>
<td>C$^2$</td>
<td>1 ND</td>
<td>1.6</td>
<td>6.4</td>
<td>1.6</td>
<td>6.4</td>
<td>6.4</td>
<td>6.4</td>
<td>ND</td>
<td>6.4</td>
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<tr>
<td></td>
<td>2 ND</td>
<td>6.4</td>
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</tr>
<tr>
<td>I-300$^3$</td>
<td>1 ND</td>
<td>ND</td>
<td>6.4</td>
<td>1.6</td>
<td>6.4</td>
<td>1.6</td>
<td>1.6</td>
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<td>6.4</td>
<td>1.6</td>
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<td>ND</td>
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<td>6.4</td>
<td>1.6</td>
<td>6.4</td>
<td>6.4</td>
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<td>$10^6$</td>
<td>$10^6$</td>
<td>$10^6$</td>
<td>$10^6$</td>
<td>$10^6$</td>
<td>$10^6$</td>
<td>$10^5$</td>
<td>$10^6$</td>
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$^1$ The data represent the value and magnitude; for example, the CYP1A2 mRNA abundance of Rat No. 1 fed on the C diet was $1.6 \times 10^6$ copies/ng of total RNA. ND: Not detected.
$^2$ C, control diet.
$^3$ I-300, control diet supplemented with FSBE as 300 mg of isoflavone/kg of diet.
$^4$ I3C, control diet supplemented with 2.5 g of indole 3-carbinol/kg of diet.
abundance of CYPs determined by quantitative competitive RT-PCR and shows. The mRNA abundance of cyclophilin from all the rats was at an equal level. The mRNA of CYP1A1 was only detected in the rats fed on the I3C diet, and the mRNA of CYP1A2 was only detected in the rats fed on the control diet. The mRNA of CYP3A1 was detected in all the rats, the mRNA abundance of CYP3A1 being above the detection limit (1.0 × 10^5 copies/ng total RNA) in the rats fed on the I3C diet. The mRNA of CYP4A1 was detected in the rats fed on the control and I3C diet. Soy isoflavone and I3C did not affect the mRNA abundance of the other isozymes.

**Experiment 2**

Table 3 shows the body weight gain, food intake, food efficiency and liver weight of the male and female rats. The dietary isoflavone decreased the food intake and body weight gain of the female rats in a dose-dependent manner, but did not affect the other parameters. Table 4 shows the relative gene expression of the isozymes of CYP in the male and female rats. The gene expression of CYP 2C11 and CYP 3A2 from the female rats was at a much lower level than that of the male rats. Dietary isoflavone significantly decreased the gene expression of CYP 3A2 and decreased the gene expression of CYP 2C11 in the female rats, although there was no significant difference among the groups (P = 0.056775). Dietary isoflavone did not significantly affect the gene expression of the other CYPs in the male and female rats.

**Discussion**

The dietary isoflavone had no inducible effect on the hepatic mRNA abundance of the major isozymes of CYPs that were determined in the present study, whether evaluated by quantitative competitive RT-PCR or real-time-monitored RT-PCR (Tables 2 and 4). This result indicates that dietary isoflavone did not cause the induction of CYPs in either the transcriptional step or post-transcriptional mRNA stabilization. There is a little information about the effect of isoflavone on the hepatic CYP activity and content. Helsby et al. have reported that genistein and equol, a metabolite of daidzein, did not affect the protein content or activity of CYP1A1, CYP1A2, CYP3A1 and CYP2E1 from a daily intraperitoneal injection to mice until 40 mg/kg of body weight.18) The soy isoflavone used in our previous study was the same as that in the present study, and daidzein and genistein likewise did not affect the total CYP content in mice.19) The amount of injected isoflavone was nearly equal to the amount of the daily isoflavone intake in the present study. It is well known that flavonoids modified CYP activity in different ways and caused various related biological effects that were either beneficial or disadvantageous to human health.11) Such CYPs as CYP1A1 and CYP1A2 oxidatively activate the precursor of carcinogens, and flavonoids decreased or increased the activity of these CYPs by the transcriptional step or post-transcriptional mRNA stabilization.10) Flavonoids might dramatically affect the plasma concentration of pharmaceutical drugs, resulting in either an
overdose or loss of their therapeutic effect, and these changes would also be expected consequence of CYP induction and/or the modulation of CYP activity by flavonoids. The activity of the isozymes of CYPs is known to be up-regulated mainly by the transcriptional step or post-transcriptional mRNA stability. However, the activity of CYP2E1 was mainly regulated by post-transcriptional protein stabilization, the contribution of the transcriptional step was also significant. These data and those from the present study suggest that isoflavones might not induce hepatic CYP activity for most isozymes, as is shown with xenobiotics, although it is not implausible that an unknown direct interaction between the isoflavone and protein of CYPs could induce hepatic CYP activity. Many researchers have reported that the concurrent administration of a drug and grapefruit juice caused an overdose. The direct action of the components of grapefruit juice on the CYP3A4 protein was the major cause of this effect. CYP3A4 is the most abundant human CYP and oxidizes a diversity of substrates, including various drugs, steroids, carcinogens, and natural macrolides products. An approach other than that used in the present study is therefore needed to evaluate the effect of soy isoflavone.

The results from the present study of the experiment using quantitative competitive RT-PCR and real-time monitored RT-PCR were well matched (Tables 2 and 4). For example, the mRNA abundance relative to cyclophilin of Rat fed on the C diet was $4.91 \pm 0.95 \times 10^{-4}$. The data represent the value and magnitude; for example, the CYP1A1 mRNA abundance relative to cyclophilin for Rat fed on the C diet was $0.0033 \pm 0.0006$. Values in a row with different superscript letters are significantly different as determined by Tukey’s multiple-range test.

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>CYP1A1</th>
<th>CYP1A2</th>
<th>CYP2B1/2</th>
<th>CYP2C11</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
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<tbody>
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<td></td>
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<td></td>
<td></td>
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<tr>
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<td>4.91</td>
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<td>0.0047</td>
<td>0.0015</td>
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* Each value represents the mean±SEM, n = 6. Values in a row with different superscript letters are significantly different as determined by Tukey’s multiple-range test.

\[ C, \text{control diet.} \]
\[ \text{I-100, control diet supplemented with FSBE as 100 mg of isoflavone/kg of diet.} \]
\[ \text{I-200, control diet supplemented with FSBE as 200 mg of isoflavone/kg of diet.} \]
\[ \text{I-300, control diet supplemented with FSBE as 300 mg of isoflavone/kg of diet.} \]
in the experiment with quantitative competitive RT-PCR, although there was no significant difference in the experiment with real-time monitored RT-PCR. As far as the mRNA abundance of CYP1A2 is concerned, the value of the standard error was higher than those of the other CYPs. Helsby et al. have reported in a later study than that just described that the soy isoflavone and its metabolites significantly decreased the protein content and activity of CYP1A2. This is in accordance with our results for mRNA abundance. Helsby et al. suggested in this report that the decrease in activity of CYP1A2 was not such to explain the chemopreventive effect of soy isoflavone.

Dietary isoflavone in the present study decreased the mRNA abundance of CYP 2C11 and CYP3A2 in female rats. These isozymes are known to be male-specific genes, and the gene expression of these two isozymes shown in the present study might be related, although the level of this gene expression was very low.

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References


