Intestinal Bacteria Activate Estrogenic Effect of Main Constituents Puerarin and Daidzin of Pueraria thunbergiana

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To understand the relationship between the metabolites and estrogenic activity of the main isoflavones puerarin and daidzin of the rhizome of Pueraria thunbergiana (PT, family Leguminosae), PT and its isoflavones were transformed by human intestinal bacteria and their estrogenic effects were investigated. All human fecal specimens hydrolyzed puerarin and daidzin to daidzein, but their hydrolyzing activities varied depending on the individuals. All intestinal bacteria isolated from human also hydrolyzed daidzin to daidzein, but a few bacteria transformed puerarin to daidzein. When the estrogenic effect of PT, puerarin and daidzin was compared with those of their metabolites, the metabolites more potently increased proliferation of MCF-7 cells than PT, puerarin and daidzin. The metabolite daidzein also potently increased estrogen-response c-fos mRNA and PR protein expressions. These findings suggest that intestinal bacteria, which can hydrolyze puerarin and/or daidzin, may activate a potent estrogenic activity of PT.

Key words Pueraria thunbergiana; isoflavone; daidzein; intestinal microflora; estrogenic effect

Most herbal medicines are orally administered to human. Their components are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. Most components may be transformed by the intestinal bacteria before absorption from the gastrointestinal tract. Studies on the metabolism of the components by human intestinal microflora are of great importance to an understanding of their biological effects.1,2)

The rhizome of Pueraria thunbergiana (PT, family Leguminosae) has been frequently used in China, Japan and Korea and antitumor cytotoxic effects.8) Yasuda reported that the metabolite daidzein exhibited the more potent antioxidant activity due to its isoflavones.3,5—7) The rhizome of PT is used for counteracting problems associated with drinking alcohol, liver injury, bone loss and menopause and many researchers reported that these pharmacological effects may originate from its isoflavones.3,5—7)

Kim et al. reported that puerarin and daidzin are metabolized to daidzein by human intestinal microflora (Fig. 1) and the metabolite daidzein exhibited the more potent antioxidant and antitumor cytotoxic effects.8) Y. Yasuda et al. reported that, when puerarin or daidzin was orally administered to rats, glucuronate and sulfate conjugates of daidzein were detected in the urine and bile.9,10) These results suggest that these isoflavone glycosides can be metabolized to their aglycones by rat intestinal bacteria before their absorptions into the blood. Nevertheless, the relationship between the metabolites of puerarin and daidzin by intestinal bacteria and their estrogenic effects has not been thoroughly studied.

Therefore, we isolated the metabolites of puerarin and daidzin of PT by human intestinal bacteria and investigated the estrogenic effect of PT and its metabolites.

MATERIALS AND METHODS

Materials Sulforhodamine B (SRB), NP40, 17β-estradiol, Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS) and charcoal dextran stripped FBS (CD-FBS) were purchased from Sigma Chem. Co. (U.S.A.). The general anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Japan). The tryptic soy broth (TS) and other media were purchased from Difco Co. (U.S.A.). The protein assay reagent was purchased from Bio-Rad Laboratories (U.S.A.). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Co. (U.S.A.). The progesterone receptor (PR) was purchased from Santa Cruz Co. (U.S.A.). The protease inhibitor cocktail was purchased from Roche Co. (Germany). All other chemicals were of analytical reagent grade, and all solutions were used after redistillation.

Puerarin (purity, >90%) and daidzin (purity, >95%) were isolated from PT according to the previous methods.8,11) Intestinal bacteria previously isolated in our lab were used in the present study.12,13)

Isolation of Metabolite Daidzein by Human Intestinal Microflora Fresh human feces (5 g) were suspended in 100 ml of anaerobic dilution medium,14) centrifuged at 500×g for 10 min, the resulting supernatant centrifuged at 10000×g for 30 min, and then washed twice with anaerobic dilution medium. The resulting precipitate was suspended in 100 ml of 20 mM phosphate buffer (pH 7.0), containing 40 mg of puerarin or daidzin, incubated for 24 h at 37 °C, and extracted with ethyl acetate. The ethyl acetate fractions were subjected to chromatography on a silica gel column, using CHCl3–MeOH (20:1) as eluent, to isolate daidzein (4 mg; purity, 90%).

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Fig. 1. Proposed Metabolic Pathway of Puerarin and Daidzin by Human Intestinal Microflora
estradiol. The treated cells were washed in PBS, and the total dilution medium was anaerobically incubated for 6 h at 37 °C. The reaction mixture (1 ml) was taken out, adjusted to pH 2 with 0.1 N HCl, extracted with EtOAc, evaporated and assayed by HPLC.

Assay of Metabolic Activity of Isoflavones by Human Intestinal Microflora The reaction mixture containing 5 ml of 1 mM isoflavones, 2.5 ml of the above fecal microflora suspension (0.5 g wet weight/ml) and 2.5 ml of the anaerobic dilution medium was anaerobically incubated for 6 h at 37 °C. The reaction mixture (1 ml) was taken out, adjusted to pH 2 with 0.1 N HCl, extracted with EtOAc, evaporated and assayed by HPLC.

Culture of MCF-7 Cells and E-Screen Assay MCF-7 cells were maintained in DMEM containing 10% FBS. Cells were grown at 37 °C in a humidified 95% air and 5% CO2 atmosphere. The cells were washed with phosphate-buffered saline (PBS), and then cultured in phenol red-free DMEM, with 10% CD-FBS, for 2 d to eliminate any estrogenic source prior to treatment. The cultured cells (5×10^3 cells/well) were seeded in culture flasks, with the medium exchanged 24 h later. The fresh phenol red-free medium contained 5% CD-FBS and the indicated test compounds. After a further 96 h, the phenol red-free medium was exchanged again, and the cells harvested after 144 h. The viable cell density numbers were determined using the SRB assay.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) MCF-7 cells were cultured with phenol red-free DMEM containing 10% CD-FBS. After 48 h, the cells were treated with each agent or 17β-estradiol. The treated cells were washed in PBS, and collected cells lysed in lysis buffer [10 mM Tris (pH 8.0), 1.5 mM MgCl2, 1 mM DTT, 0.1% NP40 and protease inhibitor cocktail]. After centrifugation at 12000 g for 5 min, the supernatant was used as the protein fraction. An equal amount of protein (30 μg) for each sample was separated by 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane at 30 V for 2 h. The membrane was then blocked with 3% skim milk in PBS/0.05% Tween 20 for 2 h at room temperature, then incubated with rabbit anti-human polyclonal antibody to PR for 2 h. After washing with PBS, the blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with ECL kits.

RESULTS

Metabolism of Puerarin and Daidzin To investigate the metabolic activity of these two isoflavones isolated from PT by human intestinal microflora, puerarin or daidzin was incubated with human fecal suspensions for 24 h and the metabolites were investigated (Table 1). All three human fecal specimens showed puerarin-hydrolyzing activity, which varied depending on the individual and metabolized it to daidzin and/or calycosin, as previously reported. The main metabolite was daidzin (>90% of the metabolites). These specimens also potently hydrolyzed daidzin to daidzin (>90% of the metabolites) and calycosin (<10% of the metabolites), as previously reported. Therefore, we assayed the metabolic activity of puerarin and daidzin intestinal bacteria from human feces. All intestinal bacteria isolated from human intestinal microflora hydrolyzed daidzin to daidzin. However, a few intestinal bacteria, such as Eubacterium rectale A-44, Streptococcus faecium S-9 and Bifidobacterium longum H-1, weakly hydrolyzed puerarin to daidzin. Among intestinal bacteria tested, Eubacterium rectale A-44 most potently produced calycosin.

Table 1. The Activities of Human Feces and Intestinal Lactic Acid Bacteria Metabolizing Puerarin or Daidzin

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Puerarin (μmol/h/g wet weight)</th>
<th>Daidzin (μmol/h/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>to daidzin</td>
<td>to calycosin</td>
</tr>
<tr>
<td>Human feces 1</td>
<td>14.5±2.3</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Human feces 2</td>
<td>2.9±0.3</td>
<td>—</td>
</tr>
<tr>
<td>Human feces 3</td>
<td>21.5±2.5</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>Bacteroides fragilis JV-6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bacteroides stercoris HI-15</td>
<td>1.3±0.2</td>
<td>—</td>
</tr>
<tr>
<td>Bifidobacterium longum H-1</td>
<td>7.4±0.5</td>
<td>—</td>
</tr>
<tr>
<td>Bifidobacterium breve K-111</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Eubacterium rectale A-44</td>
<td>15.5±1.8</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>Lactobacillus acidophilus L-2</td>
<td>9.7±1.1</td>
<td>—</td>
</tr>
<tr>
<td>Streptococcus faecium S-9</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a) Not detected. Human fecal and bacteria suspensions were prepared according to the previous method and their activities were measured according to Materials and Methods. All experiments were performed in triplicate.
mRNA expression was induced after 17β-estradiol treatment. These isoflavones also activated the transcriptions of the c-fos gene, although the effects were not as prominent as those of 17β-estradiol; daidzein most potently activated the transcriptions of the gene. The metabolized PT extract also increased the c-fos mRNA expression level more than the non-treated one. To further examine the induction of endogenous estrogen responsive genes by these isoflavones, the PR protein level was determined using immunoblot analysis. The level was found to be increased after treatment with either 17β-estradiol or daidzein, compared to that of the vehicle treated control.

DISCUSSION

Recently PT has been used as a herbal medicine for bone loss and menopause in Korea, Japan and China.3,5,6) In Western countries, it is being used as an alternative herb for post-menopausal women. Many researchers have reported that PT and its isoflavones exhibit estrogen activity.3,5–7) These isoflavones also activate estrogen-responsive genes and regulate the growth of human breast cancer cells. Most studies focused on the estrogenic effect of daidzein, genistein, and their glycosides, however, the estrogenic effect of puerarin has not been thoroughly studied. Furthermore, puerarin is metabolized to daidzein in the intestine by intestinal bacteria. Nevertheless, the relationship between these isoflavones and their phytoestrogenic effects has not been studied.

When puerarin and daidzein were incubated with human intestinal microflora, daidzein and calycosin were observed as
previously reported,\(^1\) although the main metabolite was daidzein. Daidzin was more potently metabolized than puerarin. Particularly, puerarin-hydrolyzing activity of fecal microflora varied dependently between individuals, regardless of the previous reports that intestinal microflora are rather stable over time within individuals in the absence of disease and antimicrobial therapy.\(^{16—18}\) Kobashi et al. reported that some enzymes were significantly different between individuals, although the number and species of intestinal microbes were not significantly different.\(^{10}\) These findings suggest that intestinal microflora between individuals may be changeable by environmental factors such as diet and hormones. Nevertheless, puerarin and daidzin of PT can be mainly metabolized to daidzein in the intestine by human intestinal bacteria and absorbed into the blood to express their pharmacological actions.

Therefore, we measured the estrogenic activity of puerarin, daidzin and their metabolite daidzein by E-Screen assay. Daidzein showed a more potent estrogenic effect than either of the other two. Thus, estrogenic effects of isoflavone aglycones were more potent than those of their glycosides. PT extract metabolized by human intestinal microflora also more potently increased the proliferation of MCF-7 cells than non-metabolized PT extract. The potential of isoflavones as activators of estrogen-responsive genes, the c-fos mRNA induction in MCF-7 cells was significantly different with that of its glycosides. The positive agent, \(17\beta\)-estradiol, induced the c-fos mRNA expression. These isoflavones also activated the transcriptions of the c-fos gene, with daidzein most potently activating the transcriptions. To further examine the induction of endogenous estrogen responsive genes due to these isoflavones, the PR protein levels were determined using immunoblot analysis. The levels were increased by the treatment with either \(17\beta\)-estradiol or isoflavones, compared with that of the vehicle treated control; again, daidzein most potently induced the PR protein level. Beck et al. reported that isoflavones, genistin and genistein, activated ER-mediated transcription, without direct receptor interaction.\(^{19}\) Lehmann et al. reported that daidzein is metabolized to equol by intestinal microflora, and showed a potent estrogenic effect.\(^{20}\) In the present study, we also observed that the main metabolite of puerarin and daidzin by human intestinal microflora was daidzein. Yasuda et al. detected glucuronide acid and sulfuric acid conjugates of daidzein as metabolites in the urine of orally puerarin and daidzin-administered rats.\(^{8,10}\) They reported that the daidzein level excreted in the urine of daidzin-administered rats was higher than in puerarin-administered animals. PT contains 4.5% puerarin and 0.3% daidzin. These results suggest that the estrogenic effect of PT may be dependent on the metabolism of its isoflavone glycosides, particularly puerarin, by intestinal microflora rather than daidzin. Furthermore, the metabolite daidzein has been reported to exhibit potent biological effects such as cytotoxic, antiallergic and antioxidant effects.\(^{8,11,12}\) These pharmacological activities of daidzein were stronger than those of puerarin or daidzein.

Finally, these findings suggest that intestinal bacteria, which can hydrolyze puerarin and/or daidzin of PT, may activate a potent estrogenic activity of PT.

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**REFERENCES**