Antipyretic, Analgesic and Muscle Relaxant Activities of Pueraria Isoflavonoids and Their Metabolites From Pueraria lobata Ohwi —a Traditional Chinese Drug

Takaaki YASUDA, Miwa ENDO, Toshiyuki KON-NO, Tomoko KATO, Mariko MITSUZUKA, and Keisuke OHSAWA*

Department of Phytochemistry, Tohoku Pharmaceutical University; 4–4–1 Komatsushima, Aoba-ku, Sendai 981–8558, Japan. Received November 2, 2004; accepted March 2, 2005

We evaluated the antipyretic, analgesic, and muscle relaxant activities of Pueraria isoflavonoids and their metabolites in mice. The glycosides daidzin and genistin significantly reduced fever induced by lipopolysaccharide (LPS). Their metabolites, daidzein and p-ethylphenol, also significantly reduced fever induced by LPS. In addition, daidzin, daidzein, dihydrodaidzein, and p-ethylphenol showed analgesic activity as assessed by the acetic acid-induced writhing test. Furthermore, equol and p-ethylphenol showed muscle relaxant activity in the rotarod and horizontal wire test. These results suggest that these compounds play a major role in the therapeutic activity of Pueraria isoflavonoids.

Key words Isoflavonoid; Pueraria lobata; antipyretic activity; analgesic activity; muscle relaxant activity; metabolite

Pueraria Root, consisting of the root of Pueraria lobata Ohwi (Leguminosae), has been clinically used as an antipyretic and spasmylocytic agent in traditional Chinese medicine. Many Chinese herbal formulas contain Pueraria root as their major ingredient; most well-known being “Kakkon-To” (in Japanese), which is indicated for fever and chills with stiffness or rigidity of the neck and upper back. The constituents of Pueraria root have been studied extensively with various isoflavonoids, such as puerarin, daidzin, daidzein, genistin, and genistein, having been identified. The isoflavone O-glycoside daidzin inhibits cyclic AMP phosphodiesterase and induces differentiation in murine erythroblasts. The C-glycoside puerarin exhibits hypoglycemic activity and increases coronary artery blood flow. The spasmylocytic activity of daidzein, the aglycone of daidzin, has been demonstrated by a Magnus experiment using excised murine small intestine. On the other hand, genistein had estrogenic activity, a tyrosine kinase inhibitory action, and a histidine kinase inhibitory action. However, the active compounds in Pueraria root that produce antipyretic activity have not been identified. We previously showed that orally administered glycoside daidzin and genistin were hydrolyzed to the aglycone daidzein and genistein, respectively. Furthermore, daidzin or genistein were metabolized to dihydrodaidzein, equol or dihydrogenistein, p-ethylphenol through hydrogenation, reduction or ring-fission in vivo, respectively. Therefore, we investigated the pharmacological relationship between the antipyretic, analgesic, and muscle relaxant activities and chemical components of Pueraria root to identify the active compounds and to elucidate the empirical use.

MATERIALS AND METHODS

Chemicals Daidzin, daidzein, puerarin, genistin, genistein, p-ethylphenol and aminopyrine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tween 80 and dantrolene sodium salt were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.). Lipopolysaccharide (bacterial endotoxin) from Salmonella typhimurium and methocarbamol were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents used were of analytical grade.

Animals Male ddY mice weighing 22—26 g were obtained from Japan SLC Inc. (Hamamatsu, Japan), maintained in an environmentally controlled room (22±2°C, 55±10% relative humidity, and 12-h light dark cycle), and given free access to feed and water. They were deprived of feed, but had free access to water, 2 h prior to the experiments.

Preparation of Dihydrodaidzein, Dihydrogenistein and Equol Dihydrodaidzein: The reaction mixture from the hydrogenation of daidzein in EtOH and Pd/BaSO4 under a hydrogen atmosphere was purified by Sephadex LH-20 column chromatography and recrystallized from H2O–MeOH to give dihydrodaidzein (yield 64%).

Dihydrogenistein: The reaction mixture from the hydrogenation of genistein in MeOH and Pd/BaSO4 under a hydrogen atmosphere was purified by Sephadex LH-20 column chromatography and recrystallized from H2O–MeOH to give dihydrogenistein (yield 62%).

Equol: The reaction mixture from the hydrogenation of daidzein in AcOH and Pd/C under a hydrogen atmosphere was purified by Sephadex LH-20 column chromatography and recrystallized from H2O–MeOH to give equol (yield 53%). Identification of these prepared compounds was made by comparing their EI-MS and NMR spectral data with published values.

Antipyretic Activity Test Mice with a higher body temperature than normal mice (normothermic) were used after the subcutaneous injection of LPS (50 mg/kg). Samples were injected i.p., and rectal temperatures were measured hourly. Test samples were suspended in saline with 10% Tween 80. Control mice received only the vehicle, positive controls were treated with aminopyrine, and the experimental groups received 50 and 100 mg/kg of the test compounds. Aminopyrine was used as positive control at a dose of 50 mg/kg (i.p.).

Analgesic Activity Test Test compounds were given i.p. 15 min prior to an i.p. injection of 0.7% acetic acid (0.1 ml/10 g body weight of mouse). The number of squirms was counted in each mouse for 15 min beginning 5 min after...
the last injection. Aminopyrine was used as a positive control at a dose of 25 mg/kg (i.p.).

Muscle Relaxant Activity Test  Rotarod Test: Mice were placed on a horizontal wooden rod (diameter 3 cm) that was 23 cm above the bench and rotating at a rate of 15 rev min⁻¹. After a preliminary run of naive animals, those that did not remain on the rod for 2 consecutive minutes within a period of 5 min were discarded. Immediately before giving test samples the mice were tested once more and those that did not stay on the rod for 1 min were discarded. The mice were placed on the rotarod at 0, 15, 30, 45, 60 and 75 min after injection. The time taken for each mouse to fall off the rotarod was recorded as the endurance time. If a mouse remained on the rod for more than 10 min, then its endurance time was recorded as 10 min. Total endurance time was calculated during 75 min after test sample administration.

Horizontal Wire Test: Mice were lifted by their tails and allowed to grasp a horizontally strung wire (20 cm high, 1 mm diameter, 15 cm long) with their forepaws and then released. The trials were executed two times at 30 min after injection. The number of animals from a total of ten per treatment group that did not grasp the wire with the forepaws or actively grasp the wire with at least one hind paw within 3 s was determined. In the vehicle treatment group, this number was consistently zero. Methocarbamol and dantrolene sodium salt were used as positive controls at doses of 200 mg/kg (i.p.) and 80 mg/kg (i.p.), respectively.

Statistical Analysis  Values are expressed as the mean±S.E.M. except the horizontal wire test. The results were analyzed by analysis of variance (ANOVA), supplemented by Student’s unpaired t-test for the antipyretic and rotarod tests, Dunnett’s test for the horizontal wire test, and LSD-test for the analgesic test. Values with p<0.05 were considered significant.

RESULTS

Antipyretic Activity Test  The results of the antipyretic effects are summarized in Figs. 2 and 3. Hyperthermia was observed 14 h after LPS injection and continued throughout the test. LPS (50 mg/kg, s.c.) significantly (p<0.01) increased the rectal temperature compared to the control groups over a 6 h period during the test. Aminopyrine (50 mg/kg, i.p.) reduced rectal temperature after LPS, and the antipyretic effect continued over a 6 h period with the maximum reduction 1 h after administration (Fig. 2). In addition, a high dose (100 mg/kg, i.p.) of both glycoside daidzin and genistin significantly reduced rectal temperature 1 h after administration. Aminopyrine also reduced temperature at high dose (100 mg/kg, i.p.) and the antipyretic effect continued over a 6 h period with the maximum reduction 1 h after administration (Fig. 3). In contrast, the other test compounds had no antipyretic activity, even at the high dose (data not shown).

Analgesic Activity Test  Daidzin, daidzein, dihydrodaidzein, and p-ethylphenol significantly inhibited in a dose dependent fashion the writhing induced by 0.7% acetic acid (Fig. 4). Aminopyrine also showed analgesic activity at 25 mg/kg (i.p.). On the other hand, other test compounds were ineffective in the same test.

Muscle Relaxant Activity Test  Methocarbamol and...
dantrolene sodium, which are centrally and peripherally active skeletal muscle relaxants, were used as positive controls.

In the rotarod test, the endurance times of positive control-treated mice showed a significant difference between vehicle controls at 15, 30 and 45 min for methocarbamol (200 mg/kg, i.p.) and 15, 30, 45 and 60 min for dantrolene (80 mg/kg, i.p.), respectively (Fig. 5). In addition, a low dose (50 mg/kg i.p.) of each test compound had no muscle relaxant activity (data not shown). However, a high dose (100 mg/kg i.p.) of equol, the reductive metabolite of daidzin, daidzein and genistein, had significant muscle relaxant activity at 15, 30 and 45 min after administration, and its potency was moderate. Potent muscle relaxant activity was observed in vivo with p-ethylphenol (100 mg/kg, i.p.), the degraded metabolite of genistein. Muscle relaxant activity was present for 75 min after injection, and the potency was similar to that of the positive control. In contrast, other test compounds had no muscle relaxant activity.

In the horizontal wire test, both positive control drugs significantly reduced the number of mice grasping the horizontal wire (Fig. 6). As expected, equol (100 mg/kg, i.p.) and p-ethylphenol (100 mg/kg, i.p.) also significantly decreased the number of mice grasping the horizontal wire. On the other hand, other test compounds were ineffective in this test.

**DISCUSSION**

Pueraria root is a component of Kakkon-To, which is a traditional Chinese herbal remedy used for treating patients with the common cold or neck and shoulder stiffness/rigidity. Studies have been conducted to elucidate the pharmacologi-
Therefore, we expected these metabolites to play an although the glycosides were major ingredients of Pueraria root have not yet been identified. pyretic, analgesic, and muscle relaxant activities contained in rabbits. The spasmolytic activity of daidzein was confirmed by a Magnus experiment using excised small intestine of mice. However, the active components having antipyretic, analgesic, and muscle relaxant activities contained in Pueraria root. In previous studies, we identified six metabolites in the urine of rats orally administered Pueraria isoflavonoids. These metabolites were daidzein, genistein, dihydrodaidzein, dihydrogenistein, equol and p-ethylphenol. We also reported that the glycosides daidzin and genistin were mainly hydrolyzed to the aglycone daidzein or genistein in vivo. Furthermore, it remains to be determined if the mechanism of action for p-ethylphenol at the central level is also involved in the antipyretic effect of this compound.

We also demonstrated that equol and p-ethylphenol showed muscle relaxant activity in two animal models (rotarod and horizontal wire tests) that are predictive of muscle relaxant activity in humans. Fukuda et al. pointed out that it is preferable to detection the effect of muscle relaxant activity using these two methods.

The most important finding of the present study was that only metabolites showed muscle relaxant activity, but not the original ingredients in Pueraria root, such as daidzin, puerarin and genistin and their aglycones. Accordingly, our studies indicated that equol and p-ethylphenol were the main active compounds, and contributed to the muscle relaxant activity of Pueraria root after metabolism. Our study is the first report on the muscle relaxant activity of equol and p-ethylphenol in mice, and it remains to be determined if they act centrally or peripherally. Further studies are necessary to elucidate the mechanisms of the muscle relaxant activities of p-ethylphenol and equol.

Various traditional herbal medicine formulations are regularly used in clinical practice in Japan and China. The study of the pharmacological activity of a drug will help us better understand its mechanism of action, efficacy and safety. This information provides a scientific explanation for the efficacy of the herbal medicine composite formulations that have been used based only on empirical findings. While it might be difficult to directly correlate these observations with the antipyretic activity of Pueraria root, our results are consistent with the well-known properties of this drug in traditional Chinese medicine that are based on empirical observations.

REFERENCES