— Regular Article —

Impact of *Pueraria candollei* Root Cultures on Cytochrome P450 2B9 Enzyme and Lipid Peroxidation in Mice

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Pueraria candollei (Leguminosae) has long been used as a traditional medicine for rejuvenation, and the major constituents in the tuberous root are isoflavonoids. The effects of the root cultures of *P. candollei* (r-PC) on cytochrome P450 enzymes in mouse liver and antioxidant activity in mouse brain including gross anatomy were examined. r-PC significantly enlarged the uterus length while no change in weight was noted. The total P450 content was not significantly altered by either estradiol benzoate (ES) or r-PC. Benzyloxyresorufin *O*-dealkylase activity was significantly increased by r-PC, comparable to the increase induced by ES. Correspondingly, r-PC demonstrated a tendency to upregulate CYP2B9 mRNA expression. Furthermore, assessment of lipid peroxidation in mouse brain using the thiobarbituric acid assay demonstrated that r-PC remarkably decreased the level of malondealdehyde formation. These observations suggest r-PC as a potential alternative to ES with the extrabenefit of its antioxidant activity.

Key words —— *Pueraria candollei*, benzyloxyresorufin *O*-dealkylayion, CYP2B9, estradiol, lipid peroxidation, TBARS

INTRODUCTION

Cytochrome P450 (P450) plays an important role in the detoxification of xenochemicals such as pharmaceutical drugs and environmental contaminants. On the other hand, they often result in the activation of xenochemicals to more toxic and/or carcinogenic products.[1)] The CYP2B subfamily accounts for one of the P450 enzymes, in which CYP2B1 and CYP2B2 in rats as well as CYP2B9 and CYP2B10 in mice are major CYP2B isoenzymes expressed constitutively and inducibly.[2)] Since CYP2B is involved in the metabolism of a wide variety of endogenous substrates, such as steroids, fatty acids, and retinoids, as well as several clinically important drugs, such as phenobarbital, cyclophosphamide, and midazolam,[3)] the compound that represents major metabolic or activation pathways and for CYP2B function is of considerable importance.[4)]

Oxidative damage occurs when free radicals produced within an organism are not completely destroyed by the appropriate endogenous defense systems. Since lipid is a major component of living organisms and probably the first easy target of free radicals once produced, lipid peroxidation plays an important role in initiating and/or mediating the aging process.[5)] Lipid peroxidation by reactive oxygen species (ROS) is known to be involved in the damaging mechanism of several acute maladies such as hyperbaric oxygenation and brain ischemia as well as in chronic brain disorders such as Parkinson's and Alzheimer's diseases.[6)] The most common assay currently used as an index for lipid peroxidation in biological systems is measurement of thiobarbituric acid-reactive substances (TBARS) upon the formation of a red adduct between thiobarbituric acid (TBA) and malondialdehyde (MDA).[7)]

The traditional use of medicinal plants in different maladies, such as reproductive- and fertility-related symptoms, is widespread throughout the world, especially in Asian countries. Phytogenic

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plants are of long-term interest. *Pueraria candollei* (*P. candollei*) WALL. ex BENTH. or white kwao kruae (family Leguminosae) is a rejuvenating herb in Thai folk medicine, in which phytoestrogenic compounds including puerarin, daidzin, genistin, daidzein, and genistein are accumulated in its tuberous roots.[8] Traditional medicines are usually employed for long periods. With regard to human safety evaluation, research related to hepatic drug-metabolizing enzymes is not only leading to an understanding of drug interactions or clinical significance, but also achieving other benefit of using medicinal plants as alternative medications. The inhibitory effects of *P. candollei* var. *mirifica* (*P. mirifica*)[9] on CYP1A2, CYP2B1/2, and CYP2E1 in male Wistar rats were reported.[10] However, the impact of *P. candollei* root cultures on drug-metabolizing enzymes and their antilipid peroxidation activity have not yet been thoroughly investigated. Therefore, it is worth studying whether *P. candollei* root cultures (r-PC) affect regulation of the CYP2B enzyme, as well as demonstrating their antilipid peroxidation.

The present study the effects of r-PC on the regulatory mechanism of hepatic CYP2B expression and examined its antilipid peroxidation activity in the brain in mice. The results revealed the r-PC may be a potential medicinal plant for rejuvenation based on its ability to upregulate CYP2B9 with beneficial antilipid peroxidation.

MATERIALS AND METHODS

Chemicals —— Estradiol benzoate (ES) was purchased from Schering (Kenilworth, NJ, U.S.A.). Bovine serum albumin (BSA), reduced nicotinamide adenine dinucleotide phosphate (NADPH), benzyloxyresorufin, and resorufin were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ultrahigh purity (99.99%) carbon monoxide was a product of Messer GmbH (Sulzbach/Ts., Germany). The TaKaRa RT-PCR kit (Perfect Real Time) and SYBR Green Premix Ex Taq (Perfect Real Time) were products of TaKaRa Biomedicals Inc. (Shiga, Japan). The TaqMan Gene Expression Assays were products of Applied Biosystems (Branchburg, NJ, U.S.A.). MDA and TBA were obtained from Sigma Chemical Co. and Fluka Chemika Co. (Steinheim, Switzerland), respectively. All other laboratory chemicals were of the highest purity and from commercial suppliers.

Plant Materials —— Fresh roots of 1-month r-PC were obtained from the Plant Tissue Culture Laboratory, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. Roots of the plant were dried at 50°C and finely ground to powder before filtering through a 100-mesh sieve. The plant powder of r-PC contained puerarin 1.42 mg, daidzin 19.04 mg, genistin 2.74 mg, daidzein 0.92 mg, and genistein 0.39 mg/g dry weight, respectively, quantitatively determined using HPLC-UV (Hewlett-Packard, Agilent Technologies, Bangkok, Thailand).

Animals —— ICR mice of both sexes at 8 weeks of age were obtained from the Animal Division of Faculty of Medicines, Khon Kaen University. At all times, mice were housed on woodchip bedding in stainless-steel cages with water and commercial mouse diet supplied *ad libitum* and acclimated for at least 7 days in the housing with a 12-hr light/12-hr dark cycle under controlled temperature (22 ± 2°C) and humidity (45 ± 2%) before dosing. Animal handling and the treatment protocol were approved by the Animal Ethics Committee of Khon Kaen University (approval no. AEKKU 24/2551). Mice of each sex were subcutaneously administered ES at a dose of 0.2 mg/kg in corn oil per day once a day for 1 week or orally given r-PC 500 mg/kg in distilled water per day (equivalent to puerarin 0.71 mg, daidzin 9.52 mg, genistin 1.37 mg, daidzein 0.46 mg, and genistein 0.20 mg) consecutively daily for 1, 2, and 4 weeks, respectively. The control group was simply left untreated because the vehicles did not significantly affect P450 s.[11] The mice were decapitated 24 hr after the last treatment. Uteri from female mice were individually weighed and the length was measured. Livers were immediately excised to prepare microsomes and total RNA as described elsewhere.[2]

Assessment of P450 Content and P450-Associated Activity —— The total P450 content of hepatic microsomes was determined based on the carbon monoxide difference spectra.[12] Benzyloxyresorufin *O*-dealkylase (BROD) activity was determined as described previously.[11] In brief, the reaction mixture contained hepatic microsomes (10 mg/ml), Tris-HCl 0.1 M (pH 7.8), benzyloxyresorufin 10 mM, and NADPH 10 mM. Subsequently, the formation of resorufin was immediately analyzed using spectrofluorometry with an excitation wavelength of 530 nm and emission wavelength of 585 nm.

Quantitative Determination of Hepatic CYP2B9 mRNA Expression —— Mouse CYP2B9 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were quantified using real-time reverse transcription (RT)-PCR. Hepatic total RNA was reverse-transcribed and cDNA was then amplified under the conditions recommended by the supplier (TaKaRa Biomedicals Inc.) of the TaKaRa RT-PCR kit (Perfect Real Time) using specific TaqMan Gene Expression Assays (inventoried) for CYP2B9 (Mm00657910_m1) as well as the SYBR Premix Ex Taq (Perfect Real Time) for GAPDH, in which the forward and reverse primers were 5′-TCC ACT CAC GGC AAATTC AAC G-3′ and 5′-TAG ACT CCA CGA CAT ACT CAG C-3′, respectively. The specificity of amplification of GAPDH cDNA was confirmed with both polyacrylamide gel electrophoresis and the dissociation curve of the product. RT-PCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) with ABI Prism 7500 SDS software. The conditions of each PCR cycle were as follows: denaturation at 95°C for 35 s, and annealing and extension at 60°C for 90 s. The amplified products of CYP2B9 were detected directly by monitoring the fluorescence of the reporter dye carboxyfluorescein (FAM), for which an increase in the fluorescence signal was detected only if the target sequence was complementary to the probe and amplified by PCR.[13)]

Determination of Lipid Peroxidation by Thiobarbituric Acid Assay —— Lipid peroxidation was determined by measuring TBARS formation in the TBA assay. Brain homogenates were prepared in phosphate buffer solution and 10% (w/v) trichloroacetic acid, and then homogenates were subjected to centrifugation at 5000 rpm, 4°C for 10 min. A 1.2-ml aliquot was mixed with 1 ml of 0.8% (w/v) 2-TBA. The mixture was heated at 100°C for 15 min, and then cooled to room temperature. The formation of TBARS was immediately measured using a spectrofluorometer with an excitation wavelength of 528 nm and emission wavelength of 551 nm. MDA was used as a standard.[7)]

Statistical Analysis —— The results are expressed as mean ± S.D. for each group ($n = 5$) and analyzed by one-way analysis of variance (ANOVA) followed by the Scheffe multiple-comparison test and independent sample T test (SPSS ver. 17.0). Differences of $p < 0.05$ or $p < 0.01$ were considered to be statistically significant.

Table 1. Effects of *P. candollei* Root Cultures on Weight and Length of Female Mice Uterus

Treatment	Uterus weight[$] (mg)	Uterus length[$] (cm)
Nontreatment	160.44 ± 23.36	2.40 ± 0.19
Estradiol benzoate	207.62 ± 59.21	4.18 ± 0.48**
P. candollei root cultures		
1 week	226.40 ± 46.80	3.90 ± 0.85*
2 weeks	248.62 ± 67.49	4.00 ± 1.00*
4 weeks	200.90 ± 22.16	4.35 ± 0.53*

Note: [$]Results are expressed as mean ± S.D.; *$p < 0.05$, **$p < 0.01$ *vs.* nontreatment.

RESULTS

Change in Uterine Weight and Length in Female Mice Administered r-PC

r-PC significantly enlarged uterine length in female mice comparable to those in mice adminstered ES, while uterine weight was not markedly changed by either r-PC or ES (Table 1).

Effects of r-PC on P450 Enzyme Activity

Total P450 contents were not significantly changed in both sexes of mice by either r-PC or ES. Benzyloxyresorufin *O*-dealkylation, which has been extensively utilized as an activity probe for selectively measuring CYP2B9/10 enzymes, was performed. ES significantly induced BROD activity in both sexes of mice as previously reported.[14)] Treatment with r-PC for 2 and 4 weeks significantly increased BROD activity in male mice (Table 2).

Expression of Hepatic CYP2B9 mRNA in Male Mice Administered r-PC

In accordance with BROD activity, r-PC significantly upregulated CYP2B9 mRNA expression after administration for up to 2 weeks. The induction level of CYP2B9 mRNA expression by r-PC was comparable to that of ES after longer administration for up to 4 weeks (Fig. 1).

Effects of r-PC on the Level of Lipid Peroxidation

The effects of r-PC on the level of lipid peroxidation were examined in mouse brain using the TBA assay. r-PC significantly reduced formation of TBARS in the mouse brain from the first week of r-PC adminstration. It was noted that a longer duration of r-PC treatment resulted in lower levels of MDA formation in the mouse brain (Fig. 2).

Table 2. Effects of *P. candollei* Root Cultures on Total P450 Content and BROD Activity in Mouse Liver

Treatment	Total P450 content[$] (mmol/mg protein)		Formation of resorufin[$] (pmol/mg protein per min)	
	Male	Female	Male	Female
Non treatment	0.48 ± 0.20	0.32 ± 0.15	13.04 ± 3.74	12.57 ± 4.33
Estradiol benzoate	0.18 ± 0.17	0.23 ± 0.11	37.16 ± 11.08*	27.35 ± 4.89**
P. candollei tissue cultures				
1 week	0.35 ± 0.06	0.43 ± 0.31	15.82 ± 1.87	13.97 ± 3.29
2 weeks	0.52 ± 0.17	0.40 ± 0.12	32.74 ± 7.59*	12.46 ± 1.97
4 weeks	0.71 ± 0.24	0.25 ± 0.09	39.64 ± 5.65**	20.96 ± 6.07

Note: [$]Results are expressed as mean ± S.D.; $^{*}p < 0.05$; $^{**}p < 0.01$, *vs.* nontreatment.

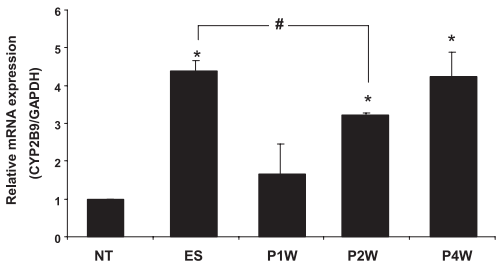

Fig. 1. Effects of *P. candollei* Root Cultures on Expression of Hepatic CYP2B9 mRNA in Male Mice

Mice were treated with subcutaneous ES daily at a dose of 0.2 mg/kg for 7 days and orally given the root powder of *P. candollei* for 1 (P1W), 2 (P2W), and 4 (P4W) weeks, respectively. Mice were decapitated 24 hr after the last treatment and the liver was immediately excised. Total RNA was prepared from the liver for determination of CYP2B9 mRNA expression as described in the Experimental section. The data are presented as mean ± S.D. (n = 5). A significant difference was determined using one-way ANOVA followed by the Scheffe multiple-comparison test. $^{*}p < 0.05$ *vs.* nontreatment (NT); $^{\#}p < 0.05$ *vs.* ES-treated group.

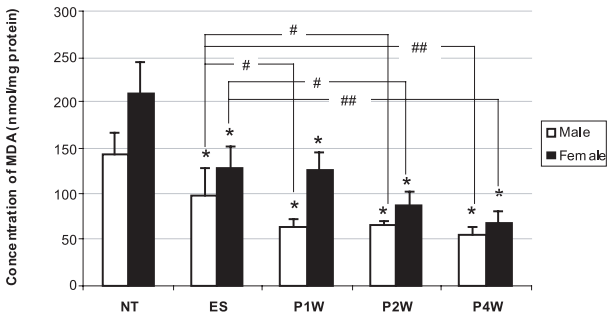

Fig. 2. Effects of *P. candollei* Root Cultures on the Level of Thiobarbituric Acid Reactive Substances in Mice

Mice were treated with subcutaneous ES daily at a dose of 0.2 mg/kg for 7 days and orally given the root powder of *P. candollei* for 1 (P1W), 2 (P2W), and 4 (P4W) weeks, respectively. Mice were decapitated 24 hr after the last treatment and the brain was excised. Brain homogenates were prepared for measuring TBARS formation as described in the Experimental section. Values are expressed as mean ± S.D. (n = 5). A significant difference was determined using one-way ANOVA followed by the Scheffe multiple comparison test. $^{*}p < 0.05$ *vs.* nontreatment (NT); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* ES-treated group.

DISCUSSION

The induction or inhibition of P450s by a natural product in the presence of a prescribed drug has led to the general acceptance that natural remedies can have some adverse effects, contrary to the popular belief in several countries where there is an active practice of ethnomedicine.[15] Phytochemical-mediated modulation of P450 activities has been widely studied.[16, 17] Since CYP2B subfamilies participate in the metabolism of endogenous substances, it is worth investigating the factors related to the expression of these genes.[2] In mice administered either ES or r-PC, the total P450 content was comparable to those in the untreated group. Benzyloxyresorufin *O*-dealkylation, which selectively measures CYP2B9/10,[2, 18] was utilized. ES significantly increased BROD activity in both sexes of mice, whereas r-PC elevated BROD levels only in male mice. A significant increase was found after 2 weeks of treatment. Correspondingly, the induction level of CYP2B9 mRNA expression by r-PC was similar to that induced by ES, with a significant increase at 4 weeks of treatment. In contrast, CYP2B10 mRNA was not significantly modified by either ES or r-PC (data not shown). These observations suggested that CYP2B9 is one of the P450 isoforms responsive to r-PC. In agreement with recent results,[14] ES upregulated the expression of CYP2B9 mRNA in the present study. The effects of r-PC on both BROD activity and CYP2B9 mRNA expression were time dependent, that is a longer period of r-PC administration induced higher levels of CYP2B9. r-PC did not change the expression level of CYP2B9 and its related enzymatic activity in females, but a marked induction was seen in the males. Because the level of CYP2B9 in male mice was lower than that in female mice,[19] greater induction was found when the expression level of

CYP2B9 was slightly changed in male mice. Although suppressive effects of *P. mirifica*[9] crude extract on hepatic BROD activity was demonstrated in rats,[10] it is impossible to compare our results with that observation since the experimental protocols was not the same. In general, CYP2B expression differes, *i.e.*, male > female in rats and female > male in mice.[20] Moreover, there are two varieties of *P. candollei*, *P. candollei* var. *candollei* and *P. mirifica*,[9] in which the phytoestrogenic contstituents are not absolutely identical, and hence we could not ensure that the same variety of *P. candollei* was employed. Besides the expression of CYP2B9, the weight and length of the uterus in female mice were observed. Mice in the r-PC group showed a significant increase in uterine length as did those in the ES group, confirming its estrogenic activity as in previous studies.[8,21] Furthermore, r-PC showed the ability to reduce the level of lipid peroxidation product in mouse brain at all durations of treatment. These observations reveal that r-PC has antioxidant activity, supporting the previous report using the diphenylpicrylhydrazyl (DPPH) assay.[22] Estradiol has been reported to be rapidly converted to 2- and 4-hydroxyestrogen by an NADPH-dependent cytochrome P450 monooxygenase system. These two estrogens have a phenolic hydroxyl group in their structure which is related to their inhibitory activity on lipid peroxidation.[23] In addition, CYP2B6 is a human CYP2B isoform mainly metabolizing 17β-estradiol, estrone, and ethinylestradiol in the human liver[24] while CYP2B9 represents a CYP2B subfamily in the mouse liver.[14]

Utilization of the r-PC as an alternative medicine should be of interest based on its potential effects on drug-metabolizing enzyme CYP2B9, estrogenic activity, and antioxidant activity. However, an in-depth risk-benefit evaluation of using r-PC and its related products is recommended. In addition, further studies focusing on the impact of individual isoflavonoid constituents of *P. candollei* on the regulatory mechanism of related hepatic P450s are required.

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