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Requirement of Metabolic Activation for Estrogenic Activity of *Pueraria mirifica*

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Abstract

A wide range of chemicals derived from plant and human-made xenobiotics are reported to have hormonal activities. The present study was performed to examine the estrogenic effect of Kwao Keur, *Pueraria mirifica* (PM), that has been used as a rejuvenating folk medicine in Thailand, using recombinant yeast, MCF-7 cell proliferation and HepG2 cell transient transfection assay. In recombinant yeast assay, 0.025, 0.25, 2.5, 25, 2.5×10^2, 2.5×10^3, 2.5×10^4 ng/ml concentrations of PM did not show any estrogenic activities, while 10^{-9} of 17 β-estradiol (positive control) showed high estrogenic activity. Estrogenic activities were induced at 2.5ng/ml to 25μg/ml concentrations of PM in a dose-dependent manner on MCF-7 cells and the estrogenic effect of PM was blocked by tamoxifen treatment, a well-known anti-estrogen. PM also showed estrogenic effect on human hepatoma cell line, HepG2 cells, containing estrogen receptor and luciferase reporter gene. Taken together, PM in itself may have no estrogenicity in yeast system, but it has estrogenicity in MCF-7 & HepG2 cells that have human metabolic enzymes. The results indicated that PM may require metabolic activation for estrogenic activity.

Key words : pueraria mirifica (PM), endocrine disrupter, metabolic activation

Introduction

The steroid hormones influence the growth, differentiation, and functioning of many target tissues. Estrogens also play an important role in bone maintenance, in central nervous system and in cardiovascular system where estrogens have certain cardioprotective effects [5, 7, 20]. Estrogen receptors (ERs) belong to the nuclear receptor superfamily, and are ligand-inducible transcription factors that mediate the biological effects of estrogens and anti-estrogens. Two ER subtypes, encoded by different genes have been isolated in mammals, ERα and ERβ [6, 9, 19]. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that ERβ is highly expressed in prostate and ovary [9, 16], but moderate expression was detected in other tissues including testis and uterus, some of which also seem to express ERα [10]. In the presence of estrogen or estrogen-like ligands, a conformational change in the ER is induced, an event that promotes ER homodimerization and high-affinity binding of ER to specific sites on DNA. Once bound to DNA, the estrogen-responsive genes, results in tissue-specific estrogenic responses.

Human diet contains several plant-derived, nonsteroidal weakly estrogenic compounds [8]. Chemically, the phytoestrogens may be divided into three main classes; flavonoids (genistein, naringenin, and kaempferol); coumestans (coumestrol); and lignans (enterodiol and enterolactone) [11]. Phytoestrogens act as weak mitogens for breast tumor cells *in vitro*, compete with 17β-estradiol for binding ER protein, and induce activity of estrogen- responsive reporter gene constructs in the presence of ER protein [12, 13, 15]. It may also act as chemopreventive agents by the fact that intake of phytoestrogens is significantly higher in countries where the incidence of breast and prostate cancers is low [14].

Pueraria mirifica (PM) is an indigenous herb of Thailand, known as "Kwao Kreu" or "Kwao Kreu Kao" (White Kwao Kreu). Similar to soybean, it belongs to the same subfamily and possesses several compounds that act as phytoestrogens like phenol miroestrol and deoxymiroestrol [3]. For over a century, the tuberous root of PM has been used by local Thai people for rejuvenating and enhancing endurance and vigor. Chansakaow *et al.* [4] reported that nine isoflavonoids including a new pterocarpene, puemiricarpe were isolated from the tuberous root of PM and showed estrogenic activity in MCF-7 human breast cancer cells.

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In the present study, estrogenic activity of PM was evaluated in recombinant yeast assay expressing human estrogen receptor (hER) and corresponding β-galactosidase reporter gene, in MCF-7 human breast cancer cells proliferation assay, and in transient transfection assay using HepG2 human hepatoma cells. Estrogenic response is created by cotransfection with recombinant rat ER α cDNA in the presence of an estrogen-dependent luciferase reporter plasmid (C3-luc).

Materials and Methods

1. Chemicals

17β-Estradiol (E2) and 4-hydroxytamoxifen (OHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *Pueraria mirifica* (PM) as test material was obtained from Cheil Jedang (In-chon, Korea). All test materials were dissolved with appropriate solvents for each experiment.

2. Recombinant yeast assay

2-1. Recombinant yeast cell

Saccharomyces cerevisiae ER+ LYS 8127 (YER) was obtained from Dr. Donald P. McDonnell (Duke University Medical Center, USA). The yeast cells were stored in 20% glycerol at -80℃. The YER cells were grown in a shaking incubator at 30℃ with 300rpm in a selective growth medium containing yeast nitrogen base (without amino acid, 67mg/ml), 1% dextrose, L-lysine (36μg/ml), and L-histidine (24μg/ml). The yeast cells were then allowed to grow until the OD values at 600nm reached between 1.0 and 2.0.

2-2. Estrogenicity assay in yeast

The yeast cells were diluted to an OD600nm value of 0.03 in selective medium plus 50 μM CuSO4 to induce receptor production. The diluted yeasts were aliquoted into 50-ml conical tube (5 ml/tube) and 5 μl of 0.025, 0.25, 2.5, 25, 250, 2.5×103 and 2.5×104 ng/ml concetrations of PM and E2 (positive control) in absolute ethanol (0.1%) were added. The cultures were incubated for 18 h in a shaking incubator at 30℃ with 300 rpm. After incubation the yeast culture samples were diluted with appropriate selective medium to an OD600nm value of 0.25 and 100 μl was added to each well of a 96-well microtiter plate. Each sample was assayed in quadruplicate. β-Galactosidase activity was induced by the addition of 100 μl of a Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl and 1 mM MgSO4, pH 7.0) containing 2 mg/ml 0-nitrophenyl-β-D-galactopyranoside (ONPG), 0.1% sodium dodecyl sulfate (SDS), 50 mM -mercaptoethanol, and 200 U/ml oxalyticase (Enzogenetics, Cornavillis, OR, USA). The OD420nm and OD590nm values of each well were measured using Titertek Multiscan MCC/344 plate reader after allowing the tube to stand for 20 min. The OD420nm value of each well was corrected by subtracting the OD590nm value.

3. MCF-7 cell proliferation assay

MCF-7 cells were grown in phenol red-free D-media (EMEM containing 50% increase of all essential amino acids except glutamine, 50% increase of all vitamins, and 100% increase of all non-essential amino acids) supplemented with 5% fetal bovine serum (FBS) and 3ml/L of PSN antibiotic mixture (Gibco, NY, USA). The cells were placed in an incubator maintained at 5% CO2, 95% air and 100% humidity at 37℃. PM was then diluted with the phenol red-free D-media supplemented with 5% dextran-coated charcoal-stripped FBS (DCC-FBS; Hyclone, UT, USA) and 3ml/L PSN antibiotic mixture (test media). The concentrating DMSO in the vehicle control media was 0.1%. E2 was used as positive control and OHT was co-treated with E2.

The cells (5×104/ml) were plated in 6-well culture plate (2ml/well) in triplicate, and allowed to attach for 24 h. The phenol red-free D-media was replaced with phenol red-free D-media supplemented with 5% DCC-FBS, followed by incubation for 24h, then the medium was removed and replaced by test medium (prepared as above) containing various concentrations of PM. The cells were incubated for 3 days at 37℃, and the test media were changed once. The cells were then washed three times with phosphate-buffered saline (PBS) and lysed with 1ml of 0.1N NaOH. The lysates were transferred into a 1.5-ml microcentrifuge tube and centrifuged for 2 minutes. The OD260nm value of the clear lysate was measured with a spectrophotometer (Du 650, Beckman, Fullerton, USA).

4. Transient transfection assay in HepG2 cell

4-1. Plating and transfection

HepG2 human hepatoma cells (Korean Cell Line Bank, Korea) were plated in triplicate in 24-well plate at a density of 5×104 cells/well in complete medium consisting of phenol red-free Eagle's minimal essential medium (GIBCO/BRL, Grand Island, NY, USA) supplemented with 10 % DCC-FBS, 2% L-glutamine, and 0.1 % sodium pyruvate. Cells were incubated for 24 h at 37℃ in a humidified atmosphere of 5 % CO2 air and then transfected following the Superfect procedure (Qiagen, Valencia, CA, USA) with two plasmids: (1) 0.4 g/ml receptor plasmid encoding rat ERα, (2) 0.8 g/well C3-luc, reporter plasmid. Transfected cells were then rinsed with PBS and treated with various concentrations of PM or with absolute alcohol (vehicle control) in complete medium. After 24 h incubation, treated cells were rinsed with PBS and lysed with 65 μl of passive lysis buffer (Promega, Madison, WI, USA). Lysate was plated into 96-well plates for luciferase determination.

4-2. Dual Luciferase reporter assay

A 100 μl volume of Lucifease assay reagent II (Promega) was added into each well containing 20 μl of lysate and then firefly luciferase activity was determined immediately using microplate luminometer LB96P (Berthold technologies, Germany). After determination of firefly luciferase activity, 100 μl of Stop & Glo reagent (Promega) was added and Renilla luciferase activity was determined. Using the DLRTM

Assay System (Promega), the luminescence from the firefly luciferase reaction is 'experimental' reporter and the luminescent reaction of Renilla luciferase is 'control' reporter.

Results

1. Recombinant yeast assay

A two-plasmid system consisting of human Estrogen receptor (hER) expression plasmid and a reporter plasmid containing estrogen response element (ERE) was employed to study estrogenic property of PM (Fig. 1). The reporter gene β-galactosidase gave a measure for ligand-dependent transactivation. In recombinant yeast assay, 0.025, 0.25, 2.5, 25, 2.5×102, 2.5×103, 2.5×104 ng/ml concentrations of PM did not induce any estrogenic activities while 10-9 of E2 as positive control had strong estrogenic activity compared with control ($p>0.05$).

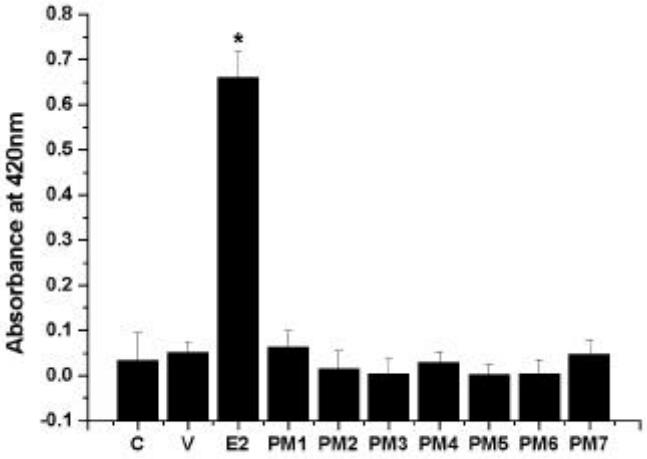

Fig. 1. Effect of *Pueraria mirifica* on the yeast expressing human estrogen receptor. C, untreated; V, vehicle (1% EtOH); E2, 10-9 M 17β-estradiol; PM1, 0.025 ng/ml; PM2, 0.25 ng/ml; PM3, 2.5 ng/ml; PM4, 25 ng/ml; PM5, 2.5×102 ng/ml, PM6, 2.5×103 ng/ml; PM7, 2.5×104 ng/ml. ∗, significantly different from control ($p>0.05$)

2. MCF-7 cell proliferation assay

Estrogenic activity of PM was estimated in terms of its proliferation-promoting effects in MCF-7 human breast cancer cells (Fig. 2). Estrogenic activity was observed significantly ($p>0.05$) compared with control from 2.5 ng/ml concentration of PM in a dose-dependent manner. The PM concentration of maximal estrogen activity was 2.5×103 ng/ml and exhibited strong proliferation similar to E2 at the concentration of 2.5 ×1010 M. DNA contents also decreased to as low as the level of vehicle control when OHT, estrogen receptor antagonist, was co-treated with PM in a dose of 2.5×103 ng/ml, maximal effective concentration of PM or 2.5×1010 M of E2, respectively.

3. HepG2 cell transient transfection assay

Estrogenic activity of PM was characterized in HepG2 human hepatoma cells transfected with rER α plus an estrogen-responsive luciferase reporter gene (Fig. 3). The estrogenic activity of PM in HepG2 cell was similar to that of PM in MCF-7 cells. PM was complete agonists at the ERα and 2.5 ×103 ng/ml of PM, maximal effective concentration and showed stronger estrogenic activity than E2 at the concentration of 10-8 M.

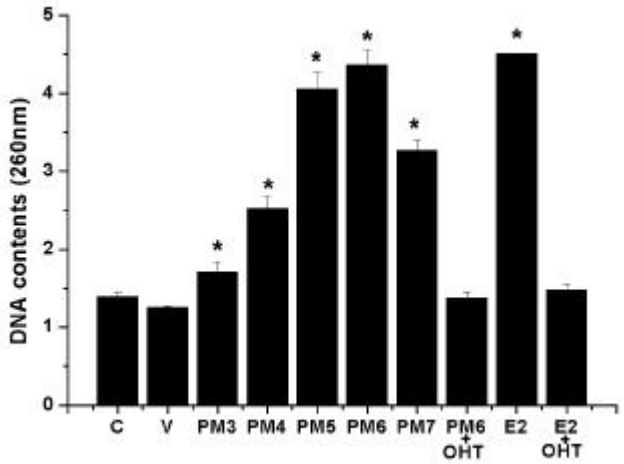

Fig. 2. Effect of *Pueraria mirifica* on the proliferation of MCF-7 human breast cancer cells. C, untreated; V, vehicle (1% EtOH); E2, 2.5×10-10 M 17β-estradiol; PM3, 2.5 ng/ml; PM4, 25 ng/ml; PM5, 2.5×102 ng/ml, PM6, 2.5103 ng/ml; PM7, 2.5×104 ng/ml; OHT, 10-6 M 4-hydroxytamoxifen. ∗, significantly different from control ($p>0.05$)

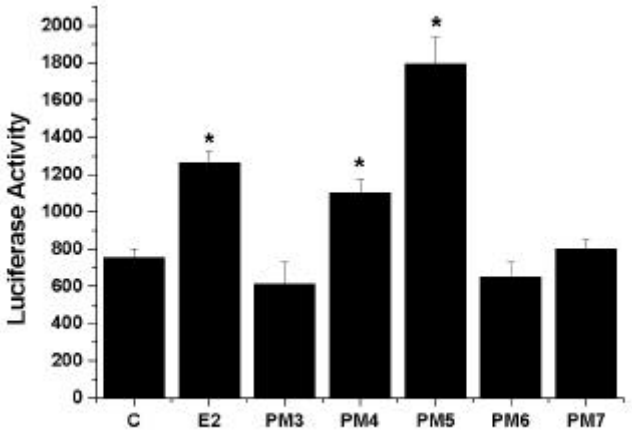

Fig. 3. Effect of *Pueraria mirifica* on the HepG2 human hepatoma cells. C, E2, 1.0×10-8 M 17β-estradiol; PM1, 0.025 ng/ml; PM2, 0.25 ng/ml; PM3, 2.5 ng/ml; PM4, 25 ng/ml; PM5, 2.5×102 ng/ml, PM6, 2.5×103 ng/ml; PM7, 2.5 ×104 ng/ml. ∗, significantly different from control ($p>0.05$)

Discussion

A wide range of chemicals derived from plant and human-made xenobiotics are reported to have hormonal activity and nowadays there are increasing tendencies to get

hormonal recipes using some natural products phytoestrogens. *Pueraria mirifica* (PM) is commonly known in Thai as White Kwao Krua, that has been used as a rejuvenating folk medicine. The enlarged underground tuber accumulates phytoestrogens comprising isoflavones such as daidzin, daidzein, genistin, genistein and puerarin. Recent studies have evaluated estrogenic activity of the isolated phytoestrogens from *Pueraria mirifica* (PM) such as kwakhurin, miroestrol, and deoxymiroestrol in MCF-7 human beast cancer cells [4].

In this study, three *in vitro* assay systems were used to evaluate the estrogenic activity of PM, and present results showed that PM did not induce estrogenic effects in recombinant yeast assay system, which PM in itself did not bind estrogen receptor. However, PM promoted MCF-7 cell proliferation in a dose-dependent manner and co-treatment PM with 4-hydroxytamoxifen inhibited PM-induced cell proliferation. Chansakaow *et al.* [4] supported our result on the estrogenic activity of PM in MCF-7 human breast cancer cell. This indicates that PM may be metabolized to a form capable of binding to the estrogen receptor in MCF-7 cells, whereas the yeast system may not have the capability to metabolically activate PM by the lack of mammalian metabolic enzymes using HepG2 human hepatoma cell would be able to know whether PM is metabolized before induction of estrogenic activity. The evaluation of PM in HepG2 cell lines confirmed that PM may be metabolized to induced estrogenic activity.

The recombinant yeast system can accurately predict the estrogenic activity of various phytoestrogens in the mammalian cell system, and it is useful for testing and detecting of novel estrogenic substances in the environment and natural specimens [1]. Also, there are many advantages of yeast system to study estrogen receptor function such as ease of manipulation, rapid attainment of stable transformants, and ability to process large sample numbers quickly and inexpensively. However, the yeast assay system cannot completely address metabolism of the compound. Some results relevant to the metabolic competence of recombinant yeast assay have been reported previously [17]. For example, Methoxychlor is metabolically converted to the active estrogenic product HPTE [2]. Shelby *et al.*[18] showed that methoxychlor (proestrogen) was inactive in the yeast assay system, whereas HPTE was active. This suggests that yeast assay system lack the ability to demethylate methoxychlor and may miss certain proestrogens, leading to negative results. HepG2 cell based system is apparently less sensitive to the action of 17β-estradiol compared to the yeast system but this system has the known properties of hepatocytes to metabolize estrogens [1]. Based on the results that PM did not induce estrogenic activity in recombinant yeast cells, but induced estrogenic activity in MCF-7 human breast cancer cells and HepG2 human hepatoma cells, therefore, PM in itself may neither bind estrogen receptor nor show estrogenic effect, but may require metabolic activation for estrogenic activity that may not be observed properly by yeast system.

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References

1. **Breithofer A., Graumann K., Scicchitano M.S., Karathanasis S.K., Butt T.R., Jungbauer A.,** Regulation of Human Estrogen receptor by phytoestrogens in Yeast and Human Cells. J. Steroid. Biochem. Mol. Biol. 1998, 67(5-6), 421-429.
2. **Bulger W.H., Muccitelli R.M., and Kupfer D.,** Studies on the in vivo and in vitro estrogenic activities of methoxychlor and its metabolites; role of hepatic monooxygenase in methoxychlor activation. Biochem. Pharmacol. 1978, 28, 2417-2423.
3. **Chansakaow S., Ishikawa T., Sekine K., Okada M., Higuchi Y., Kudo M., Chaichantipyuth C.,** Isoflavonoids from *Pueraria mirifica* and their estrogenic activity. Planta Med 2000, 66(6), 572-575.
4. **Chansakaow S., Ishikawa T., Seki H., Sekine K., Okada M., and Chaichantipyuth C.,** Identification of deoxymiroestrol as the actual rejuvenating principle of "Kwao Keur". *Pueraria mirifica*. The known miroestrol may be an artifact. J. Nat. Prod., 2000, 63(2), 173-175.
5. **Farhat M.Y., Lavigne M.C., Ramwell P.W.,** The vascular protective effects of estrogen. FASEB J 1996, 10, 615-624.
6. **Greene G.L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine Y.,** Sequence and expression of human estrogen receptor complementary DNA,. Science, 1986, 231, 1150-1154.
7. **Iafrati M.D., Karas R.H., Aronovitz M., Kim S., Sullivan T.R., Lubahn D.B., O'Donnell T.F., Korach K.S., Mendelsohn M.E.,** Estrogen inhibits the vascular injury response in estrogen receptor deficient mice. Nature Med 1997, 3, 545-548.
8. **Korach K.S., Migliaccio S., Davis V.L.,** Estrogen. In: Munson PL (ed) Principles of Pharmacology-Basic Concepts and Clinical Applications. Chapman and Hall New York 1994, 809-825.
9. **Kuiper G.G., Enmark E., Pelto-huikko M., Nilsson S. and Gustafsson J-A,** Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996, 93, 5925-5930.
10. **Kuiper G.G., Carlesson B., Grandien K., Enmark E., Haggblad J., Nilsson S., and Gustafsson J-A,** Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors and β. Endocrinology 1997, 183, 863-870.
11. **Kuiper G.G., Lemmen J.G., Carlsson B.O., Corton J.C., Safe S.H., van der Saag P.T., van der Burg B., and Gustafsson J-A,** Interaction of estrogenic

chemicals and phytoestrogens with estrogen receptor . Endocrinology 1998, 139, 4252-4263.

12. **Makela S., Davis V.L., Tally W.C., Korkman J., Salo L., Vihko R., Santti R., Korach K.S.,** Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Perspect 1994, 102, 572-581.
13. **Markiewicz L., Garey J., Adlercreutz H., Gurpide E.,** *In vitro* bioassays of non-steroidal phytoestrogens. J Steroid Biochem Mol Biol 1993, 45, 399-405.
14. **Messina M.J., Persky V., Setchell KDR, Barnes S.,** Soy intake and cancer risk: a review of the *in vitro* and in vivo data. Nutr Cancer 1994, 21, 113-131.
15. **Miksicek R.J.,** Commonly occurring plant flavonoids have estrogenic activity. Mol Pharmacol 1993, 44, 37-43.
16. **Mosselman S., Polman J., and Dijkema R.,** Identification and characterization of a novel human estrogen receptor. FEBS Lett 1996, 392, 49-53.
17. **Odum J., Lefevre P.A., Tittensor S., Paton D., Routledge E.J., Beresford N.A., Sumpter J.P. and Ashby J.,** The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Regul. Toxicol. Pharmacol. 1997, 25, 176-188.
18. **Shelby M.D., Newbold R.R., Tully D.B., Chae K., Davis V.L.,** Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays, Environ Health Perspect 1996, 104(12):1296-1300.
19. **Tremblay G.B., Tremblay A., Copeland N.G., Gilbert D.J., Jenkins N.A., Labrie F. and Giguere V.,** Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 1997, 11, 353-365.
20. **Turner R.T., Riggs B.L., Spelsberg T.C.,** Skeletal effects of estrogens. Endocr Rev 1994, 15, 275-300.