

***Pueraria Mirifica* Phytoestrogens Improve Dyslipidemia in Postmenopausal Women Probably by Activating Estrogen Receptor Subtypes**

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Impaired lipid metabolism is an important health problem in postmenopausal women with insufficient estrogens, because dyslipidemia is a risk factor for development of atherosclerosis and the incidence of cardiovascular disease markedly increases after menopause. *Pueraria mirifica* (PM), a Thai herb, has been noticed as a source of phytoestrogens, estrogen-mimicking plant compounds. However, the clinical effects of PM on lipid metabolism and the underlying molecular mechanisms remain undetermined. Therefore, we examined the effects of PM on serum lipid parameters in a randomized, double-blind, placebo-controlled clinical trial. Nineteen postmenopausal women were randomly assigned to receive oral administration of PM powder or placebo. After 2 months of treatment, the PM group showed a significant increase in serum concentrations of high-density lipoprotein (HDL) cholesterol and apolipoprotein (apo) A-1 (34% and 40%, respectively), and a significant decrease in low-density lipoprotein (LDL) cholesterol and apo B (17% and 9%, respectively), compared with baseline measurements. Moreover, significant decreases were observed in the ratios of LDL cholesterol to HDL cholesterol (37%) and apo B to apo A-1 (35%). Next, we determined the effects of PM phytoestrogens on the activation of estrogen receptor (ER)-mediated transactivation by transient expression assays of a reporter gene in cultured cells. Among PM phytoestrogens, miroestrol and coumestrol enhanced both ER α - and ER β -mediated transactivation, whereas other phytoestrogens, including daidzein and genistein, preferentially enhanced ER β -mediated transactivation. In conclu-

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sion, PM has a beneficial effect on lipid metabolism in postmenopausal women, which may result from the activation of gene transcription through selective binding of phytoestrogens to ER α and ER β . ——— *Pueraria mirifica*; Phytoestrogen; Lipid metabolism; Estrogen; Menopause.

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Lipid metabolism is an important aspect in postmenopausal women, because the incidence of cardiovascular disease markedly increases after menopause because of the progression in atherosclerosis. *Pueraria mirifica* (PM), a Thai herb, contains a large amount of phytoestrogens, estrogen-mimicking plant compounds, in its tuberous roots and has been used as a rejuvenating drug in Thailand (Ingham et al. 2002). Although a recent clinical trial showed that PM reduced perimenopausal symptoms in women (Lamlertkittikul and Chandeying 2004), no clinical trials have been conducted on the effects of PM on lipid metabolism and the underlying molecular mechanism of these effects yet remains to be fully investigated. A thorough understanding of this mechanism is needed before PM can be developed as a new therapeutic agent and clinically used without adverse effects.

Plasma levels of both high-density lipoprotein (HDL) cholesterol and its major protein component apolipoprotein (apo) A-1 are inversely correlated with the risk of coronary heart disease in population studies (Gordon et al. 1977; Castelli et al. 1986). This inverse association is important to the roles of HDL cholesterol and apo A-1 in the reverse cholesterol pathway, in which HDL cholesterol functions as an acceptor of cholesterol from the peripheral tissues and then transports it to the liver. Atherosclerosis can be modulated by raising the level of HDL cholesterol (Gordon et al. 1989) and through administration of apo A-1 (Nissen et al. 2003). More recently, development and research of Torcetrapib, one of the cholesteryl ester transfer protein (CETP) inhibitors that substantially raise the level of HDL cholesterol (Brousseau et al. 2004; Nissen et al. 2007), was terminated because of the increased mortality rate associated with its use along with a statin, as com-

pared with a group that received only statin (Pearson 2006; Singh et al. 2007). Thus, further studies are required to develop better therapeutic agents.

Estrogen exerts a wide variety of biological actions principally by binding to the estrogen receptor (ER). ER belongs to the nuclear receptor superfamily and functions as a ligand-dependent transcription factor that binds to an estrogen response element (ERE) located in the vicinity of the promoter region of target genes (Hall et al. 2001). Recent studies have established that ER requires diverse classes of multiple cofactor proteins to maximally activate target gene transcription in the presence of ligands (Rosenfeld et al. 2006). In most mammals, ER exists as two isoforms: ER α and ER β . These isoforms differ in the structure of C-terminal ligand-binding and N-terminal transactivation domains and in tissue expression (Mosselman et al. 1996). Both ER α and ER β bind 17 β -estradiol (E2) with a high affinity and stimulate transcription of the ERE-containing reporter gene in an E2-dependent manner in cell culture systems (Klinge et al. 2004). It has been reported that several phytoestrogens—including coumestrol, genistein, and daidzein—bind both ER isoforms with affinities lower than that seen with E2, and that the level of estrogenic potency of these phytoestrogens for both ER subtypes is different in transfection assays (Kuiper et al. 1998).

Miroestrol and its derivative, deoxymiroestrol, are potent estrogenic constituents purified from PM (Chansakaow et al. 2000a, b). A recent study showed that miroestrol binds to ER α in the cytosol of MCF-7 breast cancer cells and stimulates transcription of an ERE-driven reporter gene stably integrated into the genomic DNA of MCF-7 cells (Matsumura et al. 2005). However,

whether miroestrol exerts its estrogenic activity by binding not only to ER α but also to ER β remains to be examined. Moreover, whether miroestrol recruits a set of transcriptional cofactors to ER in a manner similar to that observed for E2 has not been studied. In the present study, using a transient transfection assay in CV-1 cells lacking endogenous ER expression, we compared the estrogenic potency of miroestrol with that of other phytoestrogens in the presence of cotransfection of ER α and ER β . We also evaluated whether miroestrol can recruit established cofactors such as steroid receptor cofactor-1 (SRC-1) (Onate et al. 1995) and receptor-interacting protein 140 (RIP140) (Cavaillès et al. 1995) to ER.

To clarify the effects of PM on lipid metabolism, especially HDL cholesterol and apo A-1, we conducted a randomized, double-blind, placebo-controlled clinical trial. We also investigated the molecular mechanism of the action of PM phytoestrogens *in vitro*.

MATERIALS AND METHODS

Clinical trial

This study was a randomized, double-blinded, placebo-controlled, PM intervention trial conducted at Gunma University Hospital. It was approved by the institutional review board and performed according to the Declaration of Helsinki. A test tablet (200 mg) containing 25 mg of dried PM root powder and a placebo tablet containing only the vehicle, identical to the test tablet in shape, color, odor, and taste, were prepared. Twenty-three healthy volunteers in the menopausal state were randomly divided into two groups: PM group ($n = 12$) and placebo group ($n = 11$); however, 4 subjects in the placebo group were not included in the evaluation because they had high estrogen concentrations and low FSH concentrations, which suggested that they were not menopausal. Subjects in each group were given 4 tablets daily, 2 tablets each in the morning and afternoon, for 2 months. Laboratory data and symptoms were monitored at four points: pre-administration, 1 month after administration, 2 months after administration, and 1 month after terminating administration. Fasting blood samples were obtained and written informed consent was obtained from all the subjects.

Phytoestrogens and other ligands

Miroestrol (Cain 1960; Chansakaow et al. 2000a), coumestrol, daidzein, and genistein were isolated from dried PM root powder, which contained 20 mg/kg of miroestrol and about 1 mg/kg or less of isoflavonoids (genistein, daidzein, and coumestrol) (Chansakaow et al. 2000a, b), and dissolved in dimethyl sulfoxide (DMSO). 17 β -Estradiol (E2), tri-iodothyronine (T3), *all-trans* retinoic acid (atRA) (all from Sigma-Aldrich Japan KK, Tokyo, Japan), and dihydrotestosterone (DHT) (Wako Pure Chemical Industries Ltd., Osaka, Japan) were dissolved in ethanol.

Cell culture

CV-1 cells, established from the kidney of a monkey, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin (100 μ g/ml) at 37°C in a 5% CO₂ atmosphere.

Expression vectors and luciferase reporter genes

Herpes simplex virus thymidine kinase (TK) promoter-driven luciferase reporter plasmids (TKpA₃Luc) carrying the estrogen response element (ERE-TKLuc), palindromic thyroid hormone response element (PAL-TKLuc), androgen response element (ARE-TKLuc), and retinoic acid response element (RARE-TKLuc) were as described previously (Ishizuka et al. 2001). Expression vectors for human estrogen receptor α (pKCR₂ER α), rat estrogen receptor β 1 (pSG5ER β 1), human thyroid hormone receptor β 1 (pKCR₂TR β 1), human androgen receptor (pKCR₂AR), and human retinoic acid receptor α (pKCR₂RAR α) were as described previously (Ishizuka et al. 2001). Also, expression vectors for human steroid receptor coactivator-1 (pKCR₂SRC-1) and human receptor-interacting protein 140 (pEFRIP) were as described previously (Cavaillès et al. 1995; Ishizuka et al. 2001).

Transient transfection and luciferase assay

Twenty-four hours before transfection, CV-1 cells were placed in 6-well plates in subconfluency. Cells were transiently transfected with a luciferase reporter plasmid (1.67 μ g/well) and a receptor expression vector (0.33 μ g/well) in the absence or presence of a cofactor expression plasmid (1.67 μ g/well) using calcium phosphate precipitation method as described previously (Ishizuka et al. 2001). Sixteen hours after transfection, the medium was changed to DMEM supplemented with 10% resin/charcoal double-stripped FBS, and pre-calcu-

lated amount of the compound and vehicle were added and cells were harvested after 24 h. Luciferase assay was performed in triplicate and luciferase activity was measured using a luminometer and normalized by protein concentration as described previously (Ishizuka et al. 2001).

Statistical analysis

Data are presented as mean \pm SEM. The statistical significance of differences between the treatment and placebo groups at the same periods was determined by unpaired t-test or Mann–Whitney U-test. The Friedman test was used for comparisons of more than two groups. If the Friedman test was significant, post hoc analyses were performed with the Dunn's procedure for conducting multiple comparisons of the values obtained during each treatment period with those obtained during pre-treatment. ANOVA followed by Student-Newman-Keuls multiple comparison test was used to analyze the data of transfection experiments. Statistical analyses were performed using StatFlex version 5.0 for Windows (Artech Inc., Osaka, Japan). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Subjects characteristics

Subjects in the PM ($n = 12$) and placebo ($n = 7$) groups did not differ significantly from each other in terms of age, postmenopausal period, height, weight, body mass index (22.3 ± 2.4 vs. 22.1 ± 3.5 ; $p = 0.92$), and the biochemical tests, including lipid parameters, before treatment. Each subject completed the clinical trial according to the schedule, except for one, for whom the dosage was reduced by half from the fourth day because of breast tension. After the trial, this subject was found to belong to the PM group.

Lipid parameters and laboratory tests

Lipid parameters changed substantially in the PM group during the treatment periods (Table 1). HDL cholesterol and apo A-1 increased significantly, whereas LDL cholesterol and apo B decreased significantly. Moreover, the ratios of LDL cholesterol to HDL cholesterol and apo B to apo A-1 showed a significant decrease. Total cholesterol and triacylglycerol concentrations showed no changes (data not shown). The laboratory

measurements in which changes were observed during the treatment periods can be seen in Table 1. The level of serum follicle stimulating hormone (FSH) was significantly reduced, indicating the estrogenic action of PM phytoestrogens. A high-sensitivity C-reactive protein (CRP) increased. The level of alkaline phosphatase (ALP) and γ -glutamyltranspeptidase (γ -GTP) showed a decline (data not shown).

Comparison of E2 and phytoestrogen-dependent activation of ERE reporter gene in the presence of ER α or ER β

To investigate whether PM exerts an estrogenic activity by binding to the estrogen receptor (ER), PM-dependent activation of an estrogen response element (ERE)-driven reporter gene in the presence of cotransfected ER α was evaluated. The PM phytoestrogens were tested and their effects were compared with that of E2 using a transient transfection system in CV-1 cells lacking endogenous ER expression (Fig. 1A). In our assay system, E2 significantly and maximally transactivated reporter gene activity at 10 nM, and transactivation tended to decrease at 100 nM. Miroestrol caused similar responses. In contrast, coumestrol significantly stimulated reporter activities at 100 nM and 1 μ M, and maximal activation of the concentrations tested occurred at 1 μ M. Genistein also significantly stimulated reporter gene activity at 1 μ M. Daidzein did not activate reporter activity even at the highest concentration. Activity of the ERE-dependent reporter was not stimulated by E2 or phytoestrogens in the absence of cotransfected ER (data not shown), strongly suggesting that activation of reporter activities depended on ligand activation of the ER.

Next, to examine whether miroestrol could enhance the ERE-dependent reporter gene activity by binding to ER β which differs from ER α in the structure of the carboxyl terminal ligand-binding domain and in amino-terminal activation function (Mosselman et al. 1996), we conducted a transfection assay using the same ERE reporter gene and an expression vector for ER β . E2 significantly stimulated ER β -mediated transactivation in a dose-dependent manner and maximal activation

TABLE 1. Effects of Pueraria mirifica (PM) on lipid parameters and laboratory tests in menopausal women.

	Pretreatment	1 month	2 months	Posttreatment
HDL cholesterol (mmol/l)				
Placebo	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
PM	1.6 ± 0.1	2.0 ± 0.1***† (28)*	2.1 ± 0.1***† (34)	1.7 ± 0.1 (8)
LDL cholesterol (mmol/l)				
Placebo	3.1 ± 0.2	3.1 ± 0.1	3.3 ± 0.1	3.1 ± 0.3
PM	2.9 ± 0.3	2.3 ± 0.0***† (-17)	2.3 ± 0.2***† (-17)	2.8 ± 0.2 (-2)
Ratio of LDL cholesterol to HDL cholesterol				
Placebo	2.0 ± 0.7	2.1 ± 0.7	2.2 ± 0.7	2.1 ± 0.7
PM	1.9 ± 0.7	1.2 ± 0.3***† (-34)	1.1 ± 0.3***† (-37)	1.8 ± 0.8 (-8)
Apo A-1 (g/l)				
Placebo	1.35 ± 0.05	1.33 ± 0.08	1.41 ± 0.07	1.35 ± 0.08
PM	1.46 ± 0.06	1.87 ± 0.05***†† (30)	2.03 ± 0.07***†† (40)	1.57 ± 0.07 (8)
Apo B (g/l)				
Placebo	0.86 ± 0.08	0.85 ± 0.07	0.91 ± 0.08	0.87 ± 0.07
PM	0.86 ± 0.04	0.76 ± 0.05* (-11)	0.77 ± 0.04* (-9)	0.90 ± 0.07 (3)
Ratio of apo B to apo A-1				
Placebo	0.64 ± 0.07	0.65 ± 0.06	0.66 ± 0.07	0.66 ± 0.07
PM	0.61 ± 0.05	0.41 ± 0.03***† (-30)	0.39 ± 0.03***† (-35)	0.59 ± 0.07 (-4)
FSH (IU/l)				
Placebo	63 ± 14	59 ± 14	64 ± 14	53 ± 12
PM	71 ± 3	36 ± 4**	36 ± 3**	65 ± 4
CRP (mg/l)				
Placebo	0.27	0.42	0.25	0.36
PM	0.31	0.74	0.83**	0.26

PM ($n = 12$), placebo ($n = 7$).

Values of CRP are geometric mean and other values are mean ± SEM.

‡Numbers in parentheses are percentage changes from pretreatment values in the PM group.

* $P < 0.05$; ** $P < 0.01$; Dunn's test was used for multiple comparisons of the values at each treatment period with those at pretreatment.

† $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$; Mann-Whitney's U-test was used for comparison between the PM and the placebo groups at each period.

of the concentrations tested occurred at 100 nM (Fig. 1B). Similarly, dose-dependent activation by miroestrol was observed in the presence of ER β and 100 nM of miroestrol gave maximal activa-

tion in the concentrations tested, but the activation at 10 nM was more prominent than that of E2. In contrast, coumestrol significantly activated reporter activity at 100 nM and 1 μ M, and showed

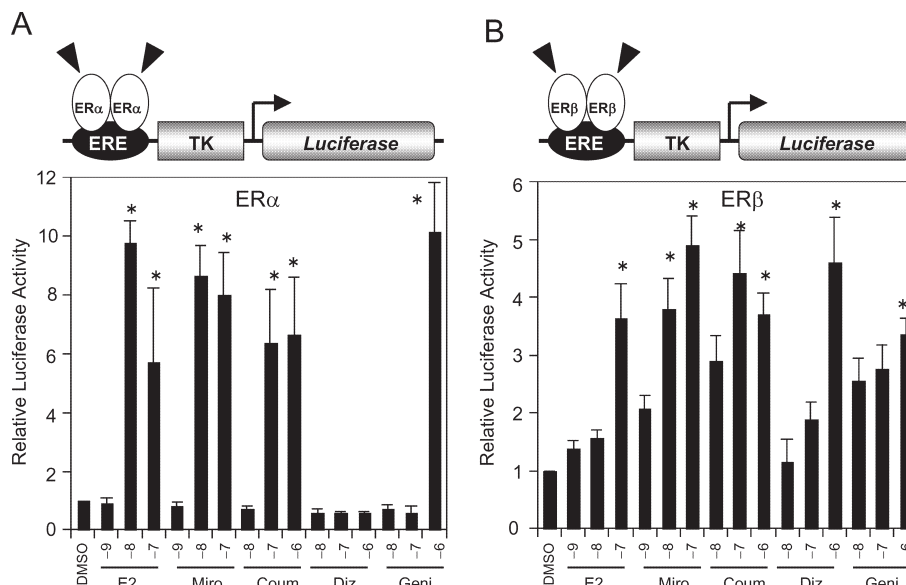


Fig. 1. Comparison of E2 and phytoestrogen-dependent activation of ERE reporter gene in the presence of ER α or ER β . ERE-TKLuc was transiently expressed in CV-1 cells with a vector expressing ER α or ER β by the calcium phosphate precipitation method. Sixteen hours after transfection, the indicated concentrations (logM) of E2, miroestrol (Miro), coumestrol (Coum), daidzein (Diz), or genistein (Geni) were added. Luciferase assay was performed after 24 h and promoter activities were expressed as relative luciferase activities (the ratio of luciferase activity with to that without a ligand). Data are mean \pm SEM from three independent transfections, in which measurements in each were made in triplicate. The structure of ERE-TK Luc, a luciferase vector carrying an estrogen response element (ERE) upstream of the herpes simplex virus thymidine kinase (TK) promoter, is shown schematically above the graphs. * $P < 0.05$ vs. DMSO.

maximal activation at 100 nM. Both daidzein and genistein significantly stimulated reporter activities at 1 μ M. These findings suggest that in the present assay system, E2 preferentially enhanced ER α -mediated transactivation; miroestrol and coumestrol enhanced both ER α - and ER β -mediated transactivation; and daidzein and genistein preferentially enhanced ER β -mediated transactivation. Notably, miroestrol and E2 enhanced ER α -mediated transactivation with the same potency.

Miroestrol did not induce transactivation mediated by other nuclear receptors

We next examined whether miroestrol-dependent transactivation could be observed in reporter gene activation mediated by other nuclear receptors. Miroestrol (100 nM) did not stimulate TR β 1-, AR-, or RAR α -mediated gene activation, whereas cognate ligands (10 nM T3, 10 nM DHT, or 100 nM atRA) significantly activated each

reporter activity (Fig. 2), indicating the specific stimulation of ER-mediated transactivation by miroestrol.

SRC-1 and RIP140 could enhance or repress ER α -mediated transactivation in the presence of miroestrol

To evaluate whether miroestrol could recruit coactivator or corepressor to ER α in living cells, we examined the effect of cotransfection with the coactivator SRC-1 (Onate et al. 1995) and the ligand-dependent corepressor RIP140 (Cavailles et al. 1995) in a transient transfection assay. Cotransfection of SRC-1 significantly potentiated ER α -mediated transactivation in the presence of 10 nM E2, and promoter activity was significantly enhanced in the presence of 100 nM miroestrol (Fig. 3A). In contrast, E2 significantly attenuated ER-mediated transactivation in the presence of cotransfected RIP140 and the same magnitude of

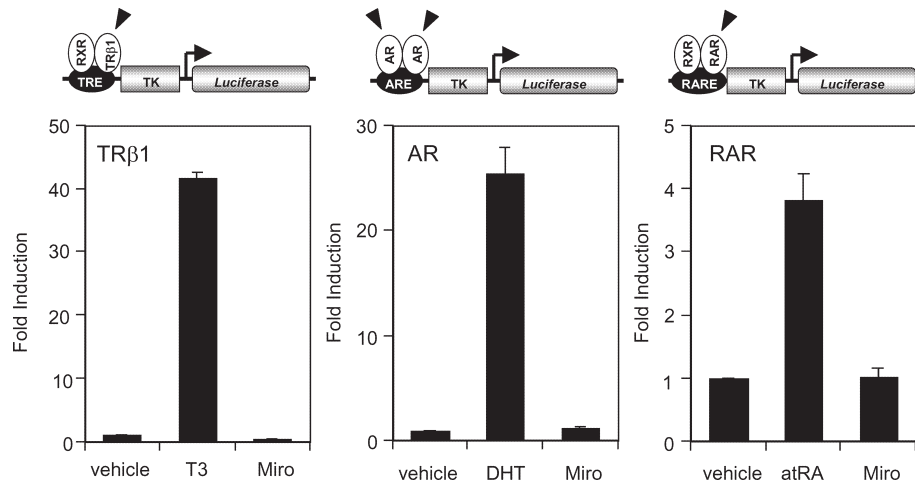


Fig. 2. Miroestrol did not stimulate transactivation mediated by other nuclear receptors. TRE-, ARE-, or RARE-TKLuc reporter gene was expressed in CV-1 cells with expression vectors for TR β 1, AR, or RAR α , respectively. Sixteen-hour after the transfection, 100 nM miroestrol, cognate ligand (10 nM T3, 10 nM DHT, or 100 nM atRA), or vehicle was added and the luciferase assay was performed as described previously. The promoter activities were expressed as relative luciferase activities (the ratio of luciferase activity with to that without a ligand). Data are mean \pm SEM from three independent transfections, in which measurements in each were made in triplicate. The structures of TRE-, ARE-, and RARE-TK Luc, the luciferase vectors respectively carrying a thyroid hormone response element (TRE), an androgen response element (ARE), and a retinoic acid response element (RARE), upstream of the TK promoter, are shown schematically above the graphs.

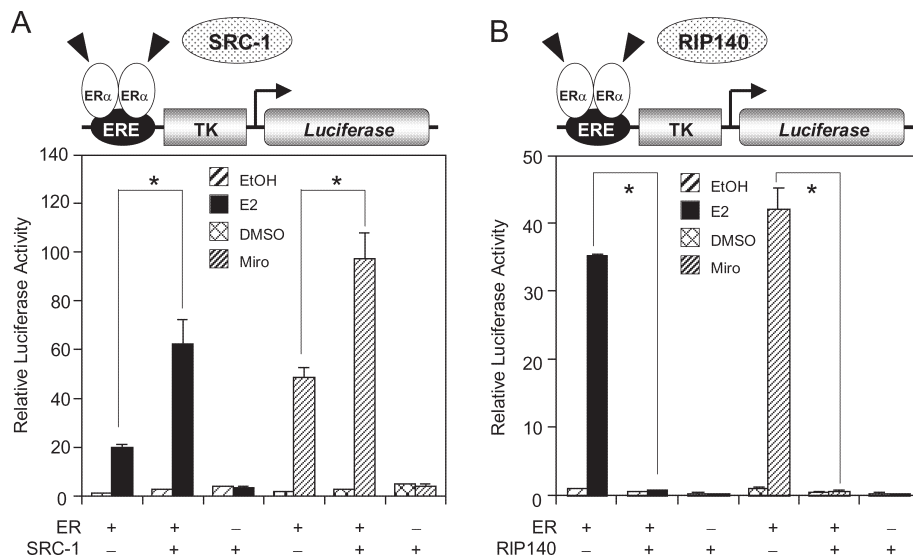


Fig. 3. SRC-1 and RIP140 could enhance or repress ER α -mediated transactivation in the presence of miroestrol. ERE-Luc was coexpressed with ER α expression vector in the absence or presence of vector expressing SRC-1 or RIP140. Sixteen hours after the transfection, 10 nM of E2, and 100 nM of miroestrol (Miro) was added. Luciferase assay was performed after 24 h and promoter activities were expressed as relative luciferase activities (the ratio of luciferase activity with to that without a ligand). Data are mean \pm SEM from three independent transfections, in which measurements in each were made in triplicate. The structure of ERE-TK Luc is shown schematically above the graphs. Asterisks indicate significant difference ($P < 0.05$).

repression was observed in the presence of miroestrol (Fig. 3B). In the absence of transfected ER, promoter activities were not altered by overexpressed cofactor proteins (Fig. 3A and B). These findings suggest that miroestrol can recruit SRC-1 or RIP140 to ER α for enhancing or repressing gene transcription in CV-1 cells, respectively.

DISCUSSION

This study has shown that PM significantly increases serum concentrations of HDL cholesterol and apo A-1, and decreases LDL cholesterol and apo B in postmenopausal women, and that PM phytoestrogens enhance gene transcription through selective binding to ER α and ER β .

In this study, administration of PM for 2 months increased serum HDL cholesterol by 34% and apo A-1 by 40%, and decreased LDL cholesterol by 17%, apo B by 9%, the ratio of LDL cholesterol to HDL cholesterol by 37%, and the ratio of apo B to apo A-1 by 35% as compared with basal values in postmenopausal women. These changes are equivalent to those reported in a study in which statin therapy was given for 18 or 24 months, which showed an increase of 7.7% in HDL cholesterol and 6.8% in apo A-1, and a decrease of 23.5% in LDL cholesterol, of 24.4% in apoB, 26.7% in the ratio of LDL cholesterol to HDL cholesterol and of 22.7% in the ratio of apo B to apo A-1 (Nicholls et al. 2007). More specifically, the changes in HDL cholesterol and apo A-1 were greater in PM administration, while those in LDL cholesterol and apo B were greater in statin therapy. Moreover, the present results are comparable to those reported for 4 weeks of Torcetrapib treatment, in which there was an increase of 46% in HDL cholesterol and 16% in apo A-1, and a decrease of 8% in LDL cholesterol and 10% in apo B as compared with the control (Brousseau et al. 2004).

Clinical trial of PM in Thailand (Lamlertkittikul and Chandeying 2004), where it was administered at a daily dose of 100 mg for 6 months, did not show any statistically significant changes in lipid parameters. This clearly contrasts with the results of our study in which PM was administered at a daily dose of 100 mg for 2 months. The baseline

characteristics of our subjects in lipid parameters were not significantly different from those recruited in the previous study. The precise cause of the discrepancy in the results is not clear, but the difference in subject types seems to be the most important factor. The subjects included in the previous study were perimenopausal women, whereas those in our study were postmenopausal. Our subjects had higher FSH levels at baseline as compared with those in the previous study. During PM administration, no significant changes in FSH levels were detected in the previous study, but a significant decline was observed in our subjects, suggesting that PM exerts more potently an estrogenic activity in menopausal women who have a feedback elevation of FSH due to insufficient estrogen. Thus, the difference in the hormone state of estrogen may be attributable to this discrepancy; the effect of PM on lipid metabolism would be more potent in menopausal women than perimenopausal women. In fact, beneficial association between blood phytoestrogen and lipoprotein levels is reported to be dominantly evident among women with low blood estrogen levels (Bailey Merz et al. 2006). Another possible explanation for the discrepancy is the difference in the PM used. We compared the constituents of various PM plants from different districts of Thailand and found great variation in their constituents (manuscript in preparation). Different concentrations of phytoestrogens in the PM plants are expected to cause different blood phytoestrogen levels and affect the changes in the lipid profile even if the same amount of PM is administered. Because higher blood phytoestrogen levels are associated with higher HDL cholesterol and lower TG levels (Bailey Merz et al. 2006), phytoestrogen contents in the PMs are one of important determinants for the effects on lipid metabolism.

Hormone replacement therapy (HRT) probably has beneficial effects on dyslipidemia, but the effects of HRT on lipid and lipoprotein levels were affected by the route of administration and type of progesterone used (Godsland 2001; Stevenson 2007). Moreover, the use of HRT carries the risk of developing hormone-dependent cancers (Rossouw et al. 2002), which reduces

compliance with treatment regimens. As an alternative medicine for HRT, phytoestrogens have weak estrogenic action under certain circumstances and antiestrogenic effects under others. Among the phytoestrogens, soy isoflavones such as genistein and daidzein have been investigated frequently, but their effects on lipid metabolism were not superior to those of PM phytoestrogens in the present study. A meta-analysis of the effect of soy on plasma lipid levels indicated a clear effect on LDL cholesterol levels, with a mean reduction of 12.9%, and a modest effect on HDL cholesterol levels, with a mean increase of 2.4% (Anderson et al. 1995). This discrepancy of the effects may account for their difference in selecting the ER subtypes. The present study demonstrated that miroestrol is a potent activator of both ER α - and ER β -mediated transactivation, whereas coumestrol is a less potent activator, and other phytoestrogens, including genistein and daidzein, revealed preferential stimulation of ER β -mediated transactivation in our reporter gene assays. The effects of HRT on HDL cholesterol levels are modified by ER α polymorphisms (Herrington et al. 2002), suggesting the importance of ER α for the control of HDL cholesterol concentrations. In this regard, miroestrol seems to play a major role in the amelioration of lipid metabolism by PM.

Our findings on miroestrol were consistent with previous observations showing similar strong activation of ERE-dependent transactivation by miroestrol in an assay using a breast cancer cell line, and its high-affinity binding to purified ER in an *in vitro* competitive binding assay (Matsumura et al. 2005). Although the concentrations of miroestrol and E2 to produce maximal activation of reporter activities in the present study were approximately two orders of magnitude higher than those found in the previous study (Matsumura et al. 2005), these discrepancies could be explained by several ways. First, we employed CV-1 cells lacking endogenous ER expression to examine the ER-isotype preference of gene activation by miroestrol, whereas they used MCF7 breast cancer cells, which express endogenous ER (Matsumura et al. 2005). Thus, the cellular environment, including the expression levels of cofac-

tor proteins that facilitate ER-mediated gene activation, might have been different. Second, we employed transient transfection of an ERE-driven reporter and ER expression vectors, whereas they used an ERE reporter gene stably integrated into MCF7 cell genomic DNA (Matsumura et al. 2005). These differences in the experimental setting may affect the potency of miroestrol in stimulating ERE-dependent gene activation.

Nuclear receptors (NR), including the ER, require diverse classes of cofactor proteins directly interacting with NR to achieve maximal efficiency in activating transcription of the target gene (Rosenfeld et al. 2006). In general, cofactors that interact directly with NR are functionally categorized into two groups: "coactivators," which facilitate ligand-dependent gene stimulation by NR, and "corepressors," which are required for ligand-independent and dependent repression of target gene transcription by NR (Rosenfeld et al. 2006). SRC-1, which belongs to the p160 family that interacts with AF2 of the ER in the presence of agonist, was the first coactivator of NR to be identified using a yeast two-hybrid screen (Onate et al. 1995; Rosenfeld et al. 2006). RIP140, which also interacts with AF2 of the ER, represses target gene transcription in the presence of agonist, and is referred to as the ligand-dependent corepressor identified for the first time ever (Cavailles et al. 1995). In the present study, we showed that SRC-1 enhanced ER-mediated transactivation in the presence of miroestrol. In addition, miroestrol exerted ligand-dependent repression of ER-dependent transcription in the presence of cotransfected RIP140. Because the potency of miroestrol was similar with that of E2 for either SRC-1-dependent enhancement or RIP140-dependent repression of ER α -mediated reporter gene transcription, miroestrol might modulate target gene transcription *in vivo* by recruiting SRC-1 or RIP140 in specific cellular circumstances.

In this study, although CRP concentrations were within the normal range, the geometric mean level was elevated from 0.31 mg/l at baseline to 0.83 mg/l after 2 months of treatment with PM. Previous studies (Nissen et al. 2005; Ridker et al.

2005) demonstrated that a reduction in the level of CRP by statin therapy improves the clinical outcome of cardiovascular diseases independently of the reduction in serum cholesterol concentrations; statin therapy for 18 months decreased the geometric mean CRP level from 2.9 to 2.3 mg/l (Nissen et al. 2005). Although their subjects were patients with cardiovascular diseases and had a high level of CRP at baseline, our subjects were postmenopausal women without cardiovascular diseases and had a normal level of CRP at baseline. In contrast, CRP elevation induced by estrogen has been reported to be not associated with progression of atherosclerosis (Lakoski et al. 2005). Therefore, further investigation is needed to understand the precise mechanism and outcome of CRP elevation after PM administration.

In the present study, PM administration lowered ALP levels, suggesting that PM reduces bone turnover because ALP is a marker of bone turnover. Therefore, PM may be useful in preventing osteoporosis, which is a major cause of disability and increased mortality rate in older women. This notion is supported by the findings of a previous study (Prestwood et al. 2003), which showed that an ultra-low dose of micronized E2 reduces bone turnover and increases the bone density of the hip, spine, and total body. PM phytoestrogens may exert an effect similar to that of E2 through their enhancement of ER-mediated transactivation. In contrast, PM administration decreased γ -GTP levels. Previous clinical and laboratory observations have suggested that estrogen is implicated in constitutive hepatic lipid metabolism and that sufficient blockage of estrogen-mediated pathways in the liver may cause massive hepatic steatosis and non-alcoholic steatohepatitis (Nemoto et al. 2002). Therefore, a decrease in γ -GTP levels may indicate an improvement in hepatic lipid metabolism mediated by the estrogenic effects of PM. Additional studies are needed to clarify the role of PM phytoestrogens in bone turnover and hepatic lipid metabolism.

In conclusion, our study indicates that PM has beneficial effects on lipid metabolism in menopausal women and the role of PM phytoestrogens in the activation of gene transcription through

selective binding to ER α and ER β . Further studies focusing on the clinical significance of PM for the treatment of dyslipidemic patients are needed.

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