Phytomedicine 19 (2012) 1332–1335

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Short communication

Suppression of BSEP and MRP2 in mouse liver by miroestrol and deoxymiroestrol isolated from *Pueraria candollei*

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ARTICLE INFO

Keywords:
Miroestrol
Deoxymiroestrol
BSEP
MRP2
Bile salt export pump

ABSTRACT

Miroestrol and deoxymiroestrol are highly active phytoestrogens isolated from the tuberous root of *Pueraria candollei* var. *mirifica* (Leguminosae). Modulatory effects of miroestrol and deoxymiroestrol on the mRNAs of BSEP and MRP2 genes involved in bile salt transportation, in C57BL/6 mice were investigated. In contrast to estradiol, miroestrol and deoxymiroestrol suppressed the expression of BSEP and MRP2 mRNA in both male and female mice. The results suggest for the first time that the use of miroestrol and deoxymiroestrol-containing products as alternative medicines or health supplements should be concerned according to their effects on key genes that regulate the bile salt export pump, which could result in the risk of hepatotoxicity and intrahepatic cholestasis.

Introduction

Miroestrol and deoxymiroestrol are phytoestrogens in the tuberous roots of *Pueraria candollei* (Chansakaow et al. 2000). Estrogenic activity of miroestrol was first investigated in rats, showing that it produced mammogenic effects (Benson et al. 1961). Miroestrol exhibited an estrogenic activity 0.25 times that of 17β-estradiol by a vaginal cornification assay (Jones et al. 1961) and deoxymiroestrol had 10-fold more potent estrogenic activity than miroestrol (Chansakaow et al. 2000). Our previous study showed that the crude extract of *Pueraria candollei* roots significantly altered the hepatic cytochrome P450s, which are drug-metabolizing enzymes (Udomsuk et al. 2010). Regarding to hepatic enzyme modifications, bile salt transportation is one of interesting factor should be alert.

Bile is a vital secretion, essential for intestinal digestion and absorption of lipids. Moreover, bile is an important route for the elimination of environmental toxins, carcinogens, drugs, and their metabolites. Bile is also a major route for the excretion of endogenous compounds and metabolic products such as cholesterol, bilirubin, and hormones (Trauner and Boyer 2003). Canalicular bile accounts for approximately 75% of the daily bile production in humans and is modified by both secretory and absorptive processes as it passes along the bile ductules and ducts. The canalicular membrane contains a bile salt export pump (BSEP) for monovalent bile salts, a conjugate export pump (MRP2) for divalent bile salts, and various other amphipathic conjugates (Saito et al. 2009). Both BSEP and MRP2 belong to the ABC superfamily. MRP2 is the major driving force for bile salt-independent bile flow, whereas BSEP drives bile salt-dependent flow (Zeleer et al. 2006). Yamamoto et al. (2006) reported that 17α-ethinylestradiol (EE2) induced hepatotoxicity by suppressing the expression of bile acid secretion in the liver (Yamamoto et al. 2006). To date, there is no report that shows the influence of miroestrol and deoxymiroestrol, which possess estrogenic-like effects, on the genes that are involved in bile salt regulation. It is of clear interest to investigate how miroestrol and deoxymiroestrol affect regulation of bile salt transportation via BSEP and MRP2 mRNA expression.

In the present study, the potential of miroestrol and deoxymiroestrol to modify the hepatic enzymes involved in bile salt transportation including BSEP and MRP2, were examined in comparison with estradiol. The results showed that both miroestrol and deoxymiroestrol significantly suppressed BSEP and MRP2 mRNA expression, whereas estradiol did not.

Abbreviations: BSEP, bile salt export pump; EE2, 17α-ethenylestradiol; ES, estradiol benzoate; RT-PCR, reverse transcription-polymerase chain reaction.

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http://dx.doi.org/10.1016/j.phymed.2012.06.007

Materials and methods

Chemicals

Miroestrol and deoxymiroestrol were isolated from tuberous roots of *P. candollei* var. *mirifica* as described previously (Chansakaow et al. 2000) and identified in comparison with the authentic standards of miroestrol and deoxymiroestrol provided by Dr. Chaiyo Chaichantipyuth, Chulalongkorn University, Bangkok, Thailand. Estradiol benzoate (ES) was purchased from Schering (Kenilworth, NJ, USA). TRIZOL®, and dNTP mixture were supplied by Invitrogen® (Carlsbad, CA, USA). ReverTraAce® and Illustra® Hot Start Master Mix were products of Toyobo®, Osaka, Japan, and GE Healthcare, Bucks, UK, respectively. Random primers and RNase inhibitor were obtained from Takara Bio Inc. (Shiga, Japan). The forward and reverse primers and the product size of the genes were as follows: (1) BSEP, 5′-CAC ACA AAG CCC CTA CCA GT-3′/5′-CCA GAG GCA GCT ATC AGG AC-3′, 231 bp; (2) MRP2, 5′-GCA CTG TAG GCT CTG GGA AG-3′/5′-CAT TTC CAA GTC TGG GAG GA-3′, 224 bp; and (3) GAPDH, 5′-TCC ACT CAC GGC AAA TTC AAC G/TAG ACT CCA CGA CAT ACT CAG C-3′, 145 bp. All others chemicals were of the highest available purity from commercial suppliers.

Animals

Both sexes of C57BL/6 mice at 6 weeks of age were supplied by the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. At all times, mice were housed on wood chip bedding in stainless-steel cages with water and commercial mouse diet supplied ad libitum and acclimated for at least 7 days in housing with a 12-h dark light cycle under controlled temperature ($22 \pm 2\,^{\circ}C$) before dosing. Animal handing and the treatment protocol were approved by the Animal Ethic Committee of Khon Kaen University, Khon Kaen, Thailand (Approval No. AEKKU42/2552). Mice were subcutaneously administered with estradiol, miroestrol, or deoxymiroestrol in corn oil at a dose of 0.5 mg/kg/day once a day for 7 days. The control group was subcutaneously administered with corn oil daily for 7 days. The mice were decapitated 24 h after the last treatment. Liver was immediately excised for preparing total RNAs as described elsewhere (Jarukamjorn et al. 1999).

Expression of MRP2 and BSEP mRNA

Mouse MRP2, BSEP, and GAPDH mRNAs were semi-quantified by RT-PCR. Hepatic total RNA was reverse-transcribed using ReverTraAce® reverse transcriptase, and then cDNA was amplified under the conditions recommended by the supplier of Illustra® Hot Start Master Mix. The conditions of PCR cycle were followed by the method of Green et al. (2000) with some modifications. After separation of the PCR products by 2% agarose gel electrophoresis, the target cDNA were detected under ultraviolet light in the presence of ethidium bromide and semi-quantified by Syngene gel documentation (Ingenius L, Cambridge, UK) and the GeneTools match program.

Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) followed by LSD post hoc test (SPSS ver. 17.0). $p \leq 0.05$ was considered statistically significant.

Results and discussion

One of the most important functions of the liver is the continuous formation of bile (Nathason and Boyer 1991). This complex process is impaired after partial hepatectomy and results in transient cholestasis with diminished overall bile flow and increased serum bile acid levels (Fukano et al. 1985). However, whether the cholestasis is caused by a transient loss of the liver-specific functions during regeneration remains unknown. In general, bile formation depends on the generation of osmotic gradients within the bile canaliculus by specific bile acid and organic anion transporters (Meier 1995). BSEP and MRP2 are major transporters in the canalicular bile salt export pump and its conjugate. In this study, modification of gene-regulated bile salt excretion by miroestrol and deoxymiroestrol were examined in the livers of male and female mice at transcriptional level and compared to a synthetic female sex hormone, estradiol. ES was used as the reference compound in the present study rather than a known hepatotoxic compound, EE2, as ES is frequently used for hormone replacement therapy (HRT, Tepper et al. 1994; Rossouw et al. 2002; Patrick et al. 2005; Mueck and Seeger 2003), whereas EE2 is normally utilized as a contraceptive medicine (Cinar et al. 2012). Moreover, there are several reports noting that use of ES in HRT causes obstruction of bile duct (Trauner and Boyer 2003; Boone et al. 2006). *P. candollei* has been widely used for rejuvenation and anti-aging purposes in Thai traditional medicine (Cherdshewasart et al. 2007a; Udomsuk et al. 2011a) according to its estrogenic like effects and ability to reduce incidences of osteoporosis, cardiovascular disease, and post-menopausal symptoms (Cherdshewasart et al. 2007b). Correspondingly, miroestrol and deoxymiroestrol, two potent phytoestrogens in *P. candollei*, were reported to exert estrogenic-like effects in MCF7 cells (Chansakaow et al. 2000), on the weight and volume of female mouse uteri (Udomsuk et al. 2010), and on sex hormone synthesis pathway-related genes (Udomsuk et al. 2011b). These findings suggested these two compounds for use in HRT. Therefore, we considered that ES was more appropriate as the reference compound than EE2 for investigating whether the use of these two compounds, miroestrol and deoxymiroestrol, for HRT cause an obstruction of the bile duct via the bile salt export pump genes. The findings lead to risk-benefit assessment for use of these two compounds as alternative medication for ES in HRT. The results showed that ES did not alter BSEP and MRP2 mRNA expression whereas miroestrol and deoxymiroestrol significantly down-regulated the mRNA expression of BSEP and MRP2 (Fig. 1A and B). Since miroestrol and deoxymiroestrol are phytoestrogens, the results from this experiment are correlated with the previous study that EE2 suppressed the expression of bile acid secretion, and decreased the ATP-dependent taurocholate transport in the hepatic canalicular membrane, a finding that was proposed to be due to the impaired expression of BSEP (Yamamoto et al. 2006). In our previous studies, miroestrol and deoxymiroestrol were used at the same dose as ES (0.5 mg/kg/day for 7 days), and biopharmacological potentials similar to ES were noted, namely the modification of cytochrome P450s, genes related to the sex hormone synthesis pathway, and anti-lipid peroxidation (Udomsuk et al. 2011b, 2012). This dose of ES exerted estrogenic-like effects in monkeys (Trisomboon et al. 2005) and this dose is in the ES range recommended for HRT (0.125–0.625 mg/kg, Patrick et al. 2005). Therefore, the same dose of miroestrol, deoxymiroestrol, and ES (0.5 mg/kg/day for 7 days) were employed in the present study to compare the potential of the phytocompounds to that of ES on the regulation of BSEP and MRP2 expression to assess the risk of bile duct obstruction and benefit of using these compounds as alternatives to ES in HRT. Although there is a known indication of the estrogenic-like potential and traditional uses of *P. candollei* only in females, both genders of mice were employed in this study. Indeed estrogen is present in males, even if at a very low level. Currently, a *P. candollei* contained product is widely used in both females and transvestites. Therefore, this study was also designed to investigate the effect of miroestrol and