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Down regulation of gene related sex hormone synthesis pathway in mouse testes by miroestrol and deoxymiroestrol

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ABSTRACT

Miroestrol and deoxymiroestrol are phytoestrogens isolated from tuberous root of *Pueraria candollei* var. *mirifica*. Modulatory effects of miroestrol and deoxymiroestrol on enzymes involved in sex-hormone synthesis pathway in male C57BL/6 mice were investigated using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Miroestrol and deoxymiroestrol suppressed the expressions of 3β-HSD, 17β-HSD1, and CYP17 while CYP19 mRNA expression was slightly decreased. In addition, the expression of 17β-HSD2 was induced in correlation with those did by estradiol. These observations supported that miroestrol and deoxymiroestrol could exhibit the same effect as estradiol regarding regulation of testicular gene related sex hormone synthesis pathway.

1. Introduction

Non-prescribed remedies become increasingly popular, particularly among postmenopausal persons who in this market are the largest consumers. A phytoestrogen-contained food or food supplement is often advocated as an alternative to hormonal replacement therapy (HRT) in a woman with contraindication to the use of conventional estrogen replacement, or simply wanting a more 'natural' alternative. Phytoestrogens are plant-containing compounds with estrogen-like biological activity [1]. The use of a certain plant in traditional medicine and folklore is ascribed to its estrogenic property. For example, pomegranate was associated with fertility [2], while hops were believed by the German clergy in the Middle Ages to lower libido [3]. In Thailand, *Pueraria candollei*, has long been used in Thai traditional medicine for rejuvenation [4]. Due to its phytoestrogenic constituents, this plant expressed estrogenic-like effects on reproductive organs in many species of animal models such as mice, ovariectomized rats, and cynomolgus monkeys [5]. Miroestrol and deoxymiroestrol are potent phytoestrogens in tuberous roots of *P. candollei*. Estrogenic activity of miroestrol was firstly investigated in rat and was shown that it produced mammogenic effect [6]. Miroestrol exhibited estrogenic activity 0.25 times of 17β-estradiol by vaginal cornification assay [7] and deoxymiroestrol had 10-fold more potent estrogenic activity than miroestrol [5]. Since miroestrol and deoxymiroestrol possess similar effects and structures to estradiol, it is of clear interest to investigate their impacts on the regulation of gene-related sex hormone synthesis pathway.

Sex steroids are synthesized in testicles and ovaries before being released in the blood in order to reach all tissues to exert their actions. There are many enzymes involved in sex hormone synthesis pathway (Fig. 1). Cholesterol was converted to pregnenolone, the main precursor of all steroid hormones. Pregnenolone converted either to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) or dehydroepiandrosterone (DHEA)

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone.

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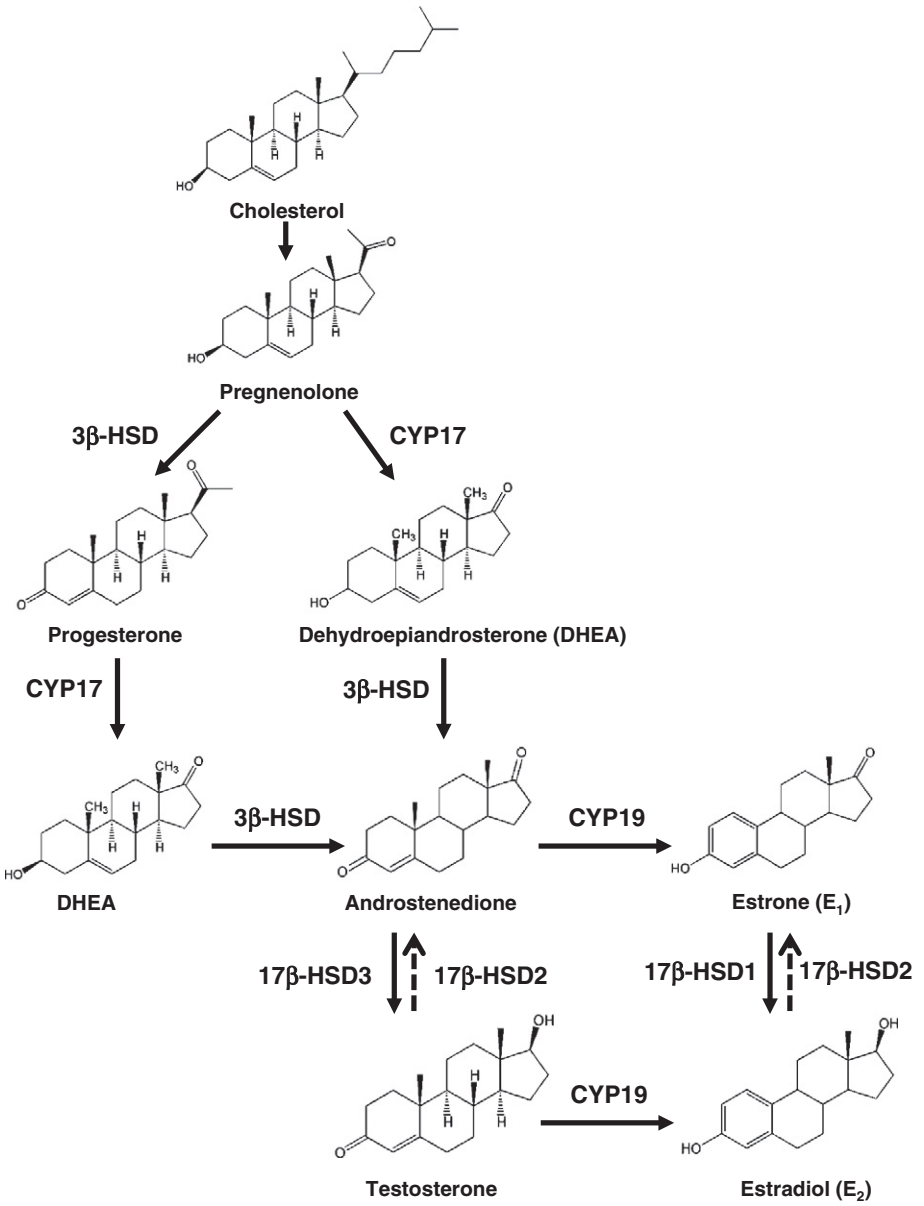

Fig. 1. Sex hormone synthesis pathway.

by CYP17 [8]. Alternatively, the products of 3β-HSD and CYP17-catalyzed reaction were metabolized by the same enzymes to form androstenedione [9]. Besides 3β-HSD and CYP17, CYP19 was involved in transformation of testosterone to estrogen [10] in various sensitive tissues. The expression of CYP19 has been suggested to be involved in breast tumor growth especially in post-menopausal patients [11]. Furthermore, potency of steroidal sex hormone like estradiol was modulated by enzymes in 17β-hydroxysteroid dehydrogenase (17β-HSD) family, especially 17β-HSD1 and 17β-HSD2, which catalyzed formation of estradiol from estrone, or conversion of estradiol to estrone, respectively [12]. 17β-HSD1 was a cytosolic enzyme present in different sex-related organs including the placenta, ovaries, breast, endometrium, and testis, which is often over-expressed in breast cancer tissue [13]. As a biological counterpart, 17β-HSD2 was responsible for inactivating the potent estrogen, estradiol, by catalyzing its conversion to estrone to protect cells from excessively high concentration of active estrogens [13].

In the present study, potential of miroestrol and deoxymiroestrol to modify testicular enzymes responsive for sex hormone synthesis including CYP17, CYP19, 3β-HSD, 17β-HSD1, and 17β-HSD2 was examined compared with estradiol. Down-regulation of genes in forwarding pathway of sex-hormone synthesis revealed that a compensatory mechanism occurred in order to lower the level of estrogen which was supplemented by miroestrol and deoxymiroestrol.

2. Experimental procedures

2.1. Chemicals

Miroestrol and deoxymiroestrol were isolated from tuberous roots of *P. candollei* var. *mirifica* as described previously [5] and identified in comparison with the authentic standards of miroestrol and deoxymiroestrol kindly provided by Dr. Chaiyo Chaichantipyuth, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Estradiol benzoate (ES) was purchased from Schering (Kenilworth, NJ, USA). TRIZOL® reagent and dNTP mixture were supplied by Invitrogen® (Carlsbad, CA, USA). Random primers and RNase inhibitor were obtained from Takara Bio Inc. (Otsu, Shiga, Japan). Forward and reverse primers of *Cyp17*, *Cyp19*, *3β-HSD*, *17β-HSD1*, *17β-HSD2*, and *GAPDH* genes (Table 1) [11,12,14,15] were synthesized by Bio Basic, Inc. (Markham Ontario, Canada). All other chemicals were of the highest available purity from commercial suppliers.

2.2. Animals

Male 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 ± 2 °C) before dosing. Animal handling and the treatment protocol were approved by the Animal Ethics 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 administrated with corn oil daily for 7 days. The mice were decapitated 24 h after the last treatment. Testes were immediately excised for preparing total RNAs as described elsewhere [16].

2.3. Semi-quantitative reverse transcription-polymerase chain reaction

Mouse CYP17, CYP19, 17β-HSD1, 17β-HSD2, 3β-HSD, and GAPDH mRNAs were semi-quantified by RT-PCR. Testicular total RNA was reverse-transcribed using ReverTraAce reverse transcriptase (Toyobo Co., Ltd.), then cDNA was amplified under the conditions recommended by the supplier of Illustra Hot Start Master Mix (GE Healthcare, UK). The conditions of PCR cycle were followed by the method of Girault et al. and Al-Soud et al. with some modifications [11,12]. After separation of the PCR products by 2% agarose gel electrophoresis, the target cDNA was 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.

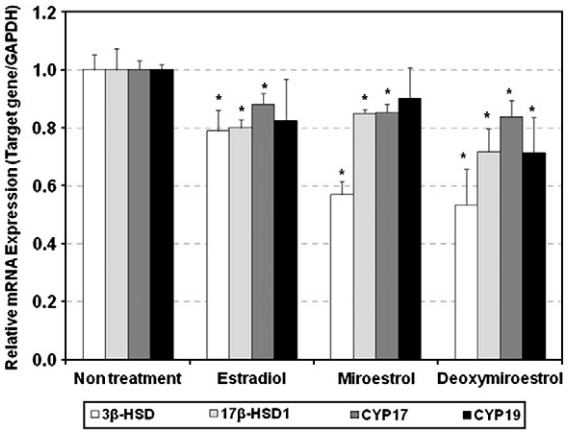

Fig. 2. Relative mRNA expressions of testicular 3β-HSD, 17β-HSD1, CYP17 and CYP19 mRNAs in male C57BL/6 mice. Mice were subcutaneously treated with estradiol, miroestrol, or deoxymiroestrol at a dose of 0.5 mg/kg body weight daily for 7 days. The data presented as mean ± SD (n = 5). *, $P<0.05$ vs non-treatment.

2.4. Statistical analysis

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

3. Results

Modification of gene-regulated sex hormone synthesis pathway including 3β-HSD, 17β-HSD1, 17β-HSD2, CYP17, and CYP19 by miroestrol and deoxymiroestrol was examined in the testes of male mice at a transcriptional level compared with a synthetic female sex hormone, estradiol. The results showed that estradiol, miroestrol, and deoxymiroestrol significantly down-regulated the mRNA expressions of 3β-HSD and CYP17, which are involved in androstenedione synthesis—testosterone and estrone precursor. 17β-HSD1, that metabolizes formation of estradiol from estrone, was significantly suppressed by miroestrol and deoxymiroestrol as those did by estradiol (Fig. 2). In addition, deoxymiroestrol decreased the level of testicular CYP19 mRNA expression whereas estradiol and miroestrol slightly decreased CYP19 mRNA (Fig. 2).

Table 1
Primer sequences for PCR.

Gene	Forward primer	Reverse primer
Cyp17	5′-CAT TCG CAC TCT GGA GTC-3′	5′-AGG CTC TTG GGG TAC TTG-3′
Cyp19	5′-CAC CCT TCC AAG TGA CAG GA-3′	5′-AAA AAA GTA AAG TTC TAT GGG AA-3′
3β-HSD	5′-CTG AAT GTT ACT GGC AAA TTC TC-3′	5′-TGTA AAA TGG ACG CAG CAG GAA-3′
17β-HSD1	5′-ACT GTG CCA GCA AGT TTG CG-3′	5′-AAG CGG TTC GTG GAG AAG TAG-3′
17β-HSD2	5′-CTG CTC AGT CTG TCC CTC CT-3′	5′-CCA GCA AAC ACT GTG AAA CC-3′
GAPDH	5′-TCC ACT CAC GGC AAA TTC AAC G-3′	5′-TAG ACT CCA CGA CAT ACT CAG C-5′

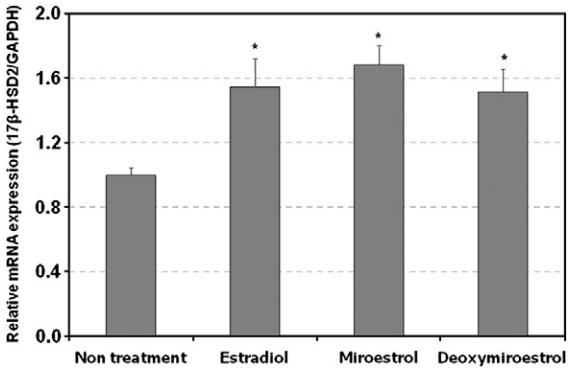

Fig. 3. Relative mRNA expression of testicular 17β-HSD2 in male C57BL/6 mice. Mice were subcutaneously treated with estradiol, miroestrol, or deoxymiroestrol at a dose of 0.5 mg/kg body weight daily for 7 days. The data presented as the mean ± SD (n = 5). *, $P<0.05$ vs non-treatment.

Expression of 17β-HSD2, an enzyme responsible for the conversion of estradiol to estrone, was further investigated. The result showed that estradiol, miroestrol, and deoxymiroestrol significantly induced the expression of 17β-HSD2 mRNA (Fig. 3). Taken together, the results suggested that miroestrol and deoxymiroestrol down-regulated the level of estrogen in the testis of male C57BL/6 mice by suppression of 3β-HSD, 17β-HSD1, CYP17 and CYP19 expressions, and induction of 17β-HSD2 mRNA expression.

4. Discussion

In female, estrogen exerts important effects on cardiovascular diseases and bone loss. Estrogen deficiency is considered to be a major factor of bone loss in postmenopausal women because estrogen inhibits bone loss by reducing bone resorption [17]. From these estrogen deficiency related problems, non-prescribed remedies are becoming increasingly popular among postmenopausal women. Phytoestrogen-containing foods, such as soy, rye, and burgen bread, and other related products, now widely available as food supplements, are becoming part of the vocabulary of patients in gynecology and menopause clinics [18]. In recent years, phytoestrogens have generated growing interest due to their potential on health benefits such as relieves of pre- and post-menopausal symptoms. There was an evidence to support the hypothesis that phytoestrogen consumption contributed to the lower incidence of cardiovascular disease in Asian countries and vegetarians [19] and that phytoestrogens may be cardioprotective [20]. Moreover, osteoporosis was related to multiple factors including aging, hormone deficiency, and diet. There were few reports demonstrating the possible role of phytoestrogens in bone metabolism and the incidence of osteoporosis [21]. For cancer prevention, consumption of phytoestrogen-rich foods such as soy, a source of isoflavones, and whole grain products, which contained lignans, showed preventive effects against breast, prostate, and colon cancers [22]. Miroestrol and deoxymiroestrol are phytoestrogens found in *P. candollei* var. *mirifica* which have been traditionally used as an alternative medicine for hormone replacement therapy [5]. Many enzymes have been involved in complex pathway of sex hormone synthesis such as 3β-HSD, CYP17, and 17β-HSDs [23]. In this experiment, the effects of miroestrol and deoxymiroestrol on gene related sex-hormone synthesis were investigated by semi-quantitative RT-PCR. The results showed that miroestrol and deoxymiroestrol down-regulated the expressions of 3β-HSD, CYP17, and 17β-HSD1 mRNAs while they up-regulated 17β-HSD2 mRNA expression as those did by estradiol. Furthermore, CYP19 was not significantly altered by estradiol, miroestrol but it was suppressed by deoxymiroestrol. There were several studies which reported the effect of *P. candollei* compared with estrogen. The crude extract of *P. candollei* exerted many biological actions like estrogen. *P. candollei* stimulated the proliferation of vaginal and uterus epithelia in rats and in women [24], inhibited the follicular growth and ovulation in female rats [25], and relieved climacteric symptoms, such as hot flushes, frustration, sleep disorder, and skin dryness in postmenopausal women [26]. From these biological activities of *P. candollei*, Chansakaow et al. [5] defined miroestrol and deoxymiroestrol as active phytoestrogens in this plant. They investigated the potency of miroestrol and deoxymiroestrol compared with estradiol using vaginal cornification assay and they reported that the estrogenic activity of miroestrol was 0.25 times of 17β-estradiol, while the growth-promoting effect of deoxymiroestrol on MCF-7 human breast cancer cells was about 10-fold more potent than miroestrol [5]. Taken all data together, both miroestrol and deoxymiroestrol affected genes in estradiol biosynthesis pathway resulting in suppression of estradiol synthesis. These findings suggested that miroestrol and deoxymiroestrol might be useful as alternative substances of estradiol or hormone replacement therapy for estrogen-like activity, due to their potential in modulating gene-related sex hormone synthesis pathway as estradiol.

Down-regulation of these enzymes including 3β-HSD, CYP17, and 17β-HSDs not only affects estradiol biosynthesis but also determines the pattern of the other steroid hormone production and their levels, which controls a variety of cellular functions. For example, 3β-HSD and CYP17 influence DHEA formation, which is in association with the aging process, vaginal atrophy, and fat accumulation [27]. In addition, suppression of 17β-HSD1 by miroestrol and deoxymiroestrol might become an extra-benefit of this compound in lowering cancer risk since over-expression of this enzyme is often related to cancer in several tissues such as breast [28] and endometriosis [29].

In conclusion, the present experiment investigated the expression of enzymes involved in the key steps of steroid hormone biosynthesis. Miroestrol and deoxymiroestrol induced the expression of 17β-HSDs family including 17β-HSD1 and 17β-HSD2 as estradiol. Furthermore, miroestrol and deoxymiroestrol suppressed the expressions of 3β-HSD and CYP17 mRNAs. The induction and suppression of these genes caused lowering synthesis of estradiol. The findings revealed that miroestrol and deoxymiroestrol might be useful for hormone replacement therapy due to their similar effects as estradiol on gene-regulated sex hormone synthesis pathway.

Conflict of interest

The authors declare no conflict of interest and declare that the research was conducted in the absence of any commercial

or financial relationships that could be construed as a potential conflict of interest.

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