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Effects of puerarin on estrogen-regulated gene expression in gonadotropin-releasing hormone pulse generator of ovariectomized rats

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ABSTRACT

Effects of puerarin on the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator function is investigated, for the first time, in ovariectomized rats at the level of mRNA expression of estrogen-responsive genes, e.g., estrogen receptor (ER), GnRH and its receptor (GnRHR). Rats were treated orally for 90 days either with a soy-free diet containing two different doses of puerarin (low dose of 600 mg/kg and high dose of 3000 mg/kg) or estradiol benzoate (E2B) at either low dose (4.3 mg/kg) or high dose (17.3 mg/kg). Levels of mRNA expression in the medial preoptic area/anterior hypothalamus (MPOA/AH), mediobasal hypothalamus/median eminence (MBH/ME) and adenohypophysis were measured by quantitative TaqMan® real-time RT-PCR. Plasma levels of luteinizing hormone (LH) and prolactin (PRL) were measured by radioimmunoassay. In the MPOA/AH, both puerarin and E2B decreased ERα mRNA levels without any significant changes in ERβ and GnRH mRNA levels. Both puerarin and E2B did not significantly alter the expression levels of ERα, ERβ and GnRHR in the MBH/ME. E2B exerted significant effects on the down-regulation of adenohypophyseal GnRHR mRNA transcripts and serum LH levels. Puerarin did not cause significant changes in pituitary GnRHR mRNA transcripts and serum LH and PRL levels. This is the first study to demonstrate that in ovariectomized rat models of ovarian hormone deprivation, puerarin acted as a weak estrogen-active compound in the hypothalamic GnRH pulse generator through the downregulation of MPOA/AH ERα mRNA expression.

1. Introduction

It is known that endogenous 17β-estradiol (E2) is essential for proper hypothalamic function. Ovarian-derived endogenous estrogen deficiency in postmenopausal females, either following the normal reproductive ageing process at the menopausal age or gonadectomy, results in the deregulation of the hypothalamic-pituitary-gonadal axis (HPGA). These effects are closely related to the impairment of the synthesis and release into the portal circulation of certain hypothalamic regulatory hormones, such as gonadotropin-releasing hormone (GnRH) that is responsible for controlling gonadotropin biosynthesis and release from adenohypophysis, and thereby an imbalance in the homeostasis of the pituitary reproductive hormones as manifested by the increased LH biosynthesis and secretion due to an overstimulation of the hypothalamic GnRH pulse generator that produces an endogenous, synchronized, pulsatile and surge pattern of GnRH release to stimulate gonadotropins secretion from the adenohypophysis [1] , which results in the typical menopausal symptom of climacteric hot flashes [2]. It has been shown that several consequences of estrogen withdrawal seen in both humans and rodents involve changes in hypothalamic neurons, including the GnRH neuron, and/or changes in pituitary responses to hypothalamic inputs [3–7]. The estrogen deficiency symptoms or diseases were in the past easily cured with the conventional hormone replacement therapy (HRT) with E2 esters, or its conjugates [8]. However, due to the severe side and confounding effects of HRT, including invasive breast and uterine cancers and cardiovascular risks, as reported by the Women's Health Initiative and Million Women Study, most physicians and patients are now uncertain whether to continue, or begin with HRT, and therefore seek alternative potential treatments instead of HRT. To date, plant-derived estrogen-active compounds (EACs), such as phytoestrogens, which structurally resemble endogenous estrogens, containing a diphenolic chemical structure that can directly bind to estrogen receptors (ER) (both subtypes of alpha (ERα) and beta (ERβ), have achieved prevalent usage as alternative substances of estrogen for HRT [2,4,6,8].

Puerarin has been characterized as the major active root-derived phytoestrogen found in Kwao Khruea Khao (*Pueraria candollei* Wall. ex Benth. var. *mirifica* (Airy Shaw & Suvat.) Niyomdham), an indigenous

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Thai herb that has been used in oriental medicine for several decades [9–11]. In vitro experiments showed that puerarin binds to both ERα and ERβ, exerts estrogen-like stimulatory effects on the proliferation of ER-responsive MCF-7 cells and displays the highest estrogenic activities through ER-dependent mechanisms of action compared to the other EACs found in the root of Kwao Khruea Khao [12-15]. In vivo data revealed that puerarin acts as a natural EAC in the peripheral and reproductive organs [9,13-17]. The above mentioned endocrine-disrupting properties mean that puerarin is more effective than the other Kwao Khruea Khao-derived EACs that can disrupt ER-regulated physiological processes, or display estrogenic potential for human's health and therapeutic beneficial aspects. However, at present there are little data available regarding whether or not dietary puerarin also acts as an EAC with the desired mechanisms of action in the brain, particularly in the neuroendocrine hypothalamus to prevent menopausal climacteric complaints. This study was therefore undertaken to investigate, in ovariectomized rats, the effects of estrogen withdrawal and replacement either with dietary estrogen, or puerarin on the changes in levels of mRNA and protein transcripts of some estrogen-responsive genes that are involved in the regulation of HPGA in the adenohypophysis, medial preoptic/anterior hypothalamic area (MPOA/AH) and mediobasal hypothalamus/median eminence area (MBH/ME). These hypothalamic brain areas are where most of the estrogen-receptive GnRH neuronal cell bodies and fibers are respectively located, and are parts of the so called "GnRH pulse generator" or "GnRH surge generator" [1,3,18], which has been reported to be implicated in the regulation of reproductive neuroendocrine function in rats [1,7,18,19].

2. Materials and methods

2.1. Test compounds

Puerarin (IUPAC Name: 7-hydroxy-3-(4-hydroxyphenyl)-8-[2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one; PubChem CID: 5281807; Molecular formula: $C_{21}H_{20}O_9$; Purity 98%) and 17beta-estradiol-3-benzoate (IUPAC Name: [(8R,9S,13S,14S,17S)-17-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthren-3-yl]benzoate; PubChem CID: 222757; Molecular formula: $C_{25}H_{28}O_3$; Purity 98.5%) was purchased respectively from Changzhou Dahua Import and Export Group, China and Sigma-Aldrich Chemicals GmbH, USA.

2.2. Animal ethics

Animal experimental design and procedures were carried out according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines and the European Detective-2010/63/EU. The animal experiments and protocols were promoted and approved by the University Medical Center Göttingen in accordance with the German animal welfare regulations (No. 509.42502/01-36.03) and the Institutional Animal Care and Use Committee of Mahasarakham University (No.0010/2012).

Virgin female Sprague-Dawley rats aged eight weeks were maintained at five to six rats per standard Makrolon type IV cage containing only sterilized wood shavings in a light, humidity and temperature controlled room (12:12-hours light and dark cycle, 22–24 °C, 50–55% relative humidity; 16 h^{-1}air change per hour) at the Animal Welfare Unit, University Medical Center, Georg-August University of Göttingen, Germany. During the maintenance and experimental periods, the animals were fed with a special phytoestrogen-free laboratory rat diet (Ssniff Spezialdiäten GmbH, Soest, Germany) and had free access to water.

2.3. Experimental design and animal treatments

At 12 weeks of age, 58 normal cycling rats, having two to three consecutive regular estrus cycles were subjected to surgical bilateral ovariectomy under a combination of xylazin and ketamin anesthesia, as previously described [15,17]. Ovariectomized rats with an average weight of 243 g were randomly allocated (n = 11–12 rats per group) into five groups: vehicle control, puerarin low, puerarin high, E2B low and E2B high. The vehicle control-treated rats were provided with the phytoestrogen-free diet without the test compounds. The puerarin low- and puerarin high-treated rats were provided with the phytoestrogen-free diet containing 0.6 g and 3 g of puerarin per kilogram diet, respectively. In the E2B low and E2B high groups, rats were given the phytoestrogen-free rat chow supplemented with E2B at doses of 0.0043 g or 0.0173 g per kilogram of chow, respectively. During a 12-week treatment interval, the animals' body weights and food weights were measured two times per week. The pathological signs and symptoms were determined daily. In this study, the chosen E2B low dose seemed suitable based on previous in vivo studies from our laboratory in which the physiological circulating concentrations of E2 and the estrogen-like actions in the uterus and pituitary gene expression were observed [4,6,15,17], whereas the E2B high dose regimen was clearly supraphysiologic [4,6]. The selected puerarin doses were due to the apparent estrogenic effects at those levels in the pituitary, uterus and vagina [9,15-17].

2.4. Euthanasia and necropsy

In this study, at the end of a 12-week treatment interval, euthanasia and necropsy were performed across all groups of rats to reduce the effects of inter-individual and time variations. The rats were rapidly exposed to carbon dioxide asphyxiation for euthanasia and then subjected to death by decapitation between 08:00 a.m. and 12:00 a.m. Blood was collected from the body and stored for two to three hours in a 4 °C refrigerator. The serum was separated by centrifugation at 4 °C, 2500 rpm for 30 min, and then kept at -20 °C for analysis of LH and PRL concentrations. The whole brains and anterior parts of pituitaries were immediately dissected out from the skull, snap frozen on dry ice for the brains and in liquid nitrogen for the anterior pituitaries, and then kept in a −80 °C ultra deep freezer. Uteri were carefully dissected without the remnant fat and were immediately weighed. All animal carcasses were also dissected for the complete removal of the ovaries.

2.5. Analysis of serum LH and PRL levels

The specific radioimmunoassays for rat LH and PRL were supported by the National Hormone and Pituitary Program of the National Institutes of Health and the National Institute of Diabetes and Digestive and Kidney Diseases (Maryland, USA) with the previously described procedures [17,20,21].

2.6. Microdissections of MPOA/AH and MBH/ME

The frozen brains were removed from storage at −80 °C and then placed and mounted on the receptacle for a freezing microtome (Leica Microsystems Nussloch GmbH, Wetzlar, Germany) using Tissue-Tek® O.C.T. (Sakura Finetek, Netherland). The microdissections of the MPOA/AH and MBH/ME were carried out as described in previous publications [22,23] in accordance to the rat brain atlas [24]. In brief, the serial frontal sectioning of the frozen brains with a thickness of 600 μm each was performed at −10 °C, thaw-mounted on a glass slide, immediately placed on dry ice and then stored in a -80 °C ultra deep freezer. The bilateral tissue micropunches of the MPOA/AH were taken from the frozen coronal brain tissue sections with a 1-mm-diameter hypodermic needle in accordance with the method of Palkovits (1973) [25]. The stereotaxic coordinates for the MPOA/AH, which refer to the rostral plane of the respective brain tissue section and the center of the needle, were A 7.8, L 0.75 and V −1.8. As described previously [26], the MBH/ME was dissected out from the respective frozen brain tissue section between the optic chiasm, hypothalamic grooves and mammillary bodies.

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