Toxicology 304 (2013) 69–75

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Effects of the natural endocrine disruptor equol on the pituitary function in adult male rats

Panida Loutchanwoot[a,b,*], Prayook Srivilai[a], Hubertus Jarry[c]

[a] Department of Biology, Faculty of Science, Mahasarakham University, Khamriang Sub-district, Kantarawichai District, Mahasarakham Province 44150, Thailand
[b] Department of Biology, Faculty of Science, Burapha University, Bangsaen Sub-district, Muang District, Chonburi Province 20131, Thailand
[c] Department of Endocrinology, University Medical Center Göttingen, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany

ARTICLE INFO

Article history:
Received 27 August 2012
Received in revised form 9 November 2012
Accepted 28 November 2012
Available online 7 December 2012

Keywords:
Endocrine disruptor
Pituitary
Equol
mRNA expression
Male rat

ABSTRACT

Equol (EQ), a potent biologically active metabolite of the soy isoflavone daidzein, interacts with estrogen receptors (ERs), however, as suggested recently, EQ may also exert anti-androgenic actions in androgen regulated tissues like prostate and seminal vesicles in adult male rats. However, data regarding a putative anti-androgenic activity of EQ on pituitary function in male individuals are still lacking. Therefore, we investigated the effects of EQ on androgen- and estrogen-regulated gene expressions in the pituitary and circulating luteinizing hormone (LH) and prolactin (PRL) levels in adult male rats. 3-Month-old male Sprague-Dawley rats ($n = 12$ per group) were treated by gavage for 5 days with either EQ (100 and 250 mg/kg BW/day) or vehicle olive oil (1 ml/rat/day). As reference compound, the pure anti-androgenic drug flutamide (FLUT) was employed at a dose of 100 mg/kg BW/day. At day 5, animals were sacrificed. Levels of pituitary hormones and gene expression were measured by radioimmunoassays and quantitative TaqMan® real-time reverse transcription polymerase chain reaction, respectively. The present findings revealed that the pituitary mechanisms involved in the effects of EQ and FLUT were different due to the opposite changes in the mRNA expression levels of estrogen receptor subtype alpha (ERα)-, truncated estrogen receptor product-1 (TERP-1)- and -2 (TERP-2)-, gonadotropin releasing hormone receptor (GnRH receptor)-, beta-subunit of LH (LHβ)-, and gonadotropin alpha subunit (α-subunit) genes. EQ displayed typical ER-agonistic actions as shown by the significant increases in ERα-, TERP-1/-2 mRNA expressions and serum PRL levels along with the significant reduction in serum LH levels, whereas FLUT exerted opposite effects on gonadotropin secretion and expression. Taken together, our findings are the first *in vivo* data that upon sub-acute oral exposure of EQ show an estrogenic effect on reproductive endocrine activity of the pituitary in adult male rats. However, EQ did not exert anti-androgenic effects on male rat pituitary function as observed at the levels of mRNA expression of androgen- and estrogen-regulated genes and circulating pituitary hormones.

1. Introduction

Numerous anthropogenic and natural plant-derived substances can mimic or disrupt the normal function of endogenous sex steroid hormones (Endocrine Disruptors, EDs). They exert their effects by direct interaction with estrogen receptors subtypes alpha (ERα) and beta (ERβ), and androgen receptor (AR) (Acerini and Hughes, 2006; Kelce and Wilson, 1997; Sonnenschein and Soto, 1998). However, an increasing number of recent studies indicate that EDs are rather promiscuous with regard to steroid receptor selectivity, *i.e.*, they may be ligands for nuclear receptors other than ERs, in particular for the AR. For example, vinclozolin, a potent anti-androgenic dicarboximide fungicide, has been shown to possess ERs-agonistic activities both *in vitro* (Kelce and Wilson, 1997; Molina-Molina et al., 2006; Scippo et al., 2004) and *in vivo* (Loutchanwoot et al., 2008). *Vice versa* the known ER agonist equol (EQ), a metabolite of the soy phytoestrogen daidzein produced *in vivo* by the intestinal bacteria (Bowey et al., 2003; Setchell and Clerici, 2010a; Yuan et al., 2007), may also acts as an anti-androgen (Hedlund et al., 2003; Lund et al., 2004, 2011; Setchell and Clerici, 2010b).

Over the last decades, much of the interest in EQ has focused on its estrogenic effects. Recent investigations utilizing *in vitro* assays, including competition-binding assays with both ERα and ERβ proteins and transcriptional activation in mammalian and yeast cell-based assays, have confirmed that EQ possesses estrogenic properties mediated through ERs (Bovee et al., 2008; Muthyala et al., 2004; Sathyamoorthy and Wang, 1997). Furthermore, utilizing cell

* Corresponding author at: Department of Biology, Faculty of Science, Mahasarakham University, Khamriang Sub-district, Kantarawichai District, Mahasarakham Province 44150, Thailand. Tel.: +66 43 754245; fax: +66 43 754245.
E-mail addresses: panida.l@msu.ac.th, oupanida@hotmail.com (P. Loutchanwoot).

0300-483X/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2012.11.017

proliferation assays, it was demonstrated that EQ stimulated in an estrogen-like manner the growth of the ER-positive human mammary adenocarcinoma MCF-7 cells (Ju et al., 2006; Liu et al., 2010; Sathyamoorthy and Wang, 1997) and of human endometrial adenocarcinoma Ishikawa cells (Lehmann et al., 2005).

In a considerable number of *in vivo* studies EQ increased the uterine weight along with a dose-dependent increase of uterine epithelial proliferation and endometrial thickness in adult ovariectomized mice, rats and monkeys, but with a much lesser extent than 17β-estradiol (E2) (Rachoń et al., 2007b; Selvaraj et al., 2004; Wood et al., 2006). EQ fed neonatally *via* the dam led to precocious mammary gland differentiation, resulting in a decrease in immature terminal end structures and an increase in mature lobules (Brown et al., 2010). A mammotrophic effect of EQ was also observed in ovariectomized monkeys (Wood et al., 2006). Chronic exposure to dietary EQ stimulated mRNA expression levels of estrogen-responsive genes in the uteri and pituitaries of adult ovariectomized rats (Rachoń et al., 2007a, 2007b).

Logically, any substances which prevent androgens from expressing their activity at target sites are referred to as "anti-androgens". The inhibitory effect of these substances, therefore, would be differentiated from estrogens or compounds which decrease the synthesis and/or release of hypothalamic (releasing factors) and anterior pituitary hormones (gonadotropins, particularly luteinizing hormone) and materials which act directly on the gonads to inhibit biosynthesis and/or secretion of androgens (Neumann and Topert, 1986). For example, the potent and pure non-steroidal anti-androgen flutamide (FLUT) competed with testosterone (T) and 5α-dihydrotestosterone (DHT) for binding to the AR, and blocked the recognition of T and DHT. The relative weights of accessory reproductive organs, *i.e.*, ventral prostate and seminal vesicles, which are androgen-dependent, were decreased by FLUT treatment. The inability of the pituitary to recognize androgens stimulated the secretion of LH by FLUT treatment (Andrews et al., 2001; Kunimatsu et al., 2004; O'Connor et al., 1998a, 2002; Ohsako et al., 2003).

Recently, EQ has been considered as a potential pharmaceutical or nutraceutical agent for androgen-dependent cancers because *in vitro* it has direct anti-proliferative effects on human benign and malignant prostatic epithelial cells at concentrations typically found in the plasma and prostatic fluid of men consuming a soy-rich diet (Hedlund et al., 2003). Utilizing the yeast androgen bioassay, Bovee et al. (2008) confirmed that EQ possesses anti-androgenic property which is most likely caused by its ability to interact with AR. However, Lund et al. (2004, 2011) reported that the *in vitro* anti-androgenic properties of EQ maybe unique since EQ does not bind to AR, but specifically binds with high affinity to DHT (but not T or E2), and thereby preventing DHT from binding to AR, and blocking the stimulatory androgen action of DHT in increasing prostatic specific antigen (PSA) levels in human cancer prostate cells. An *in vivo* anti-androgenic action of EQ on the male reproductive endocrine function was shown by the significant decrease in DHT concentrations without altering T, E2 and luteinizing hormone (LH) levels along with a significant decline in prostate and epididymis weights in adult male rats (Lund et al., 2004, 2011).

Despite the evident endocrine activity of EQ in the urogenital system of both female and male animals, little is known about its putative anti-androgenic effects in the other steroid hormone receptive organs including the pituitary. With this in mind, therefore the present study is the first attempt to investigate the putative *in vivo* anti-androgenic effects of EQ on male rat pituitary gene expression and hormone secretion in comparison to those of the reference anti-androgenic compound, FLUT. Serum levels of EQ and FLUT were determined to prove sufficient bioavailability of the test compounds. In addition, the classical endpoints suggested by the Organization for Economic Cooperation Development (OECD), *i.e.*, wet weights at autopsy of the androgen-dependent reference organs ventral prostate and seminal vesicles were also evaluated. Utilizing this experimental approach, we report the first evidence that sub-acute oral administration of EQ revealed a dose-dependent endocrine action in the pituitary of adult male rats which is most likely to be estrogenic. As demonstrated by the concomittant increased LH secretion and -expression, FLUT was proved to be a pure anti-androgen in the pituitary.

2. Materials and methods

2.1. Test substances

Equol (3,4-dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol; CAS-No. 531-95-3) was obtained from the Changzhou Dahua Imp. and Exp. (Group) Corp. Ltd. (Changzhou, Jiangsu, China). Flutamide (2-methyl-*N*-(4-nitro-3-(trifluoromethyl) phenyl)-propanamide; CAS-No. 13311-84-7) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Animals and treatments

All animals used in this study have been treated in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123). The experiments were approved by a permit issued by the Landesamt für Verbraucherschutz, Braunschweig, Germany (Aktenzeichen 33.11.42502-04-01-30.05). Male Sprague-Dawley rats at the age of 2 months were obtained from Winkelmann GmbH, Borchen, Germany. Animals were fed with soy-free chow (Ssniff Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*. They were standardized environmental conditions (room temperature 22–24 °C, relative humidity of 50–55%, illumination from 06:00 a.m. until 06:00 p.m.).

At the age of 3 months, male rats were weighed and divided by randomization into four experimental groups ($n = 12$/group), *i.e.*, control-, EQ low dose-, EQ high dose-, and FLUT-group, so that there were no statistically significant differences among the group body weight means prior to the treatment. Animals were orally treated *via* gavage once per day for 5 consecutive days with either olive oil as the vehicle control, EQ at low dose of 100 mg/kg BW/day or at high dose of 250 mg/kg BW/day. FLUT at a dose of 100 mg/kg BW/day served as anti-androgenic reference positive control. The doses of EQ selected for this study referred to those which in adult ovariectomized rats produced by an estrogen-like effect in the pituitary and uterus, but did not cause adverse effects (Rachoń et al., 2007a, 2007b). The dose of FLUT chosen based on previously published studies, showing antagonism of both peripheral- and central AR resulted in decreased sizes of androgen-dependent male accessory reproductive organs, and increased LH secretion, respectively, in adult male rats (Andrews et al., 2001; Kunimatsu et al., 2004; O'Connor et al., 2002; Yu et al., 2004). Test substances were dissolved in olive oil and applied in a volume of 1 ml per animal per day. Treatments were conducted during 8.00–9.30 a.m. During a 5-day treatment period, body weight and clinical symptoms were recorded daily.

2.3. Necropsy, collection of target organs, and measurement of organ weights

At day 5, 2 h after the last application, animals were rapidly exposed to CO_2 asphyxiation, followed by decapitation. Blood was collected from the trunk and serum samples were stored at −20 °C. The peripheral androgen receptive organs ventral prostate and seminal vesicles were dissected and immediately weighed. Pituitaries were removed from the skull and the anterior part was snap frozen in liquid nitrogen and kept at −70 °C until gene expression analysis.

2.4. Endpoint evaluation

The endpoints evaluated included (a) serum concentrations of luteinizing hormone (LH) and prolactin (PRL); (b) pituitary levels of gene expression of estrogen receptor subtypes alpha (ERα) and beta (ERβ), truncated estrogen receptor product-1 (TERP-1) and -2 (TERP-2), androgen receptor (AR), gonadotropin releasing hormone receptor (GnRH receptor), beta-subunit of LH (LHβ), and gonadotropin alpha subunit (α-subunit); and (c) wet weights at autopsy of the androgen-dependent reference organs ventral prostate and seminal vesicles.

2.5. Serum hormone analysis

Levels of LH and PRL were measured using rat specific radioimmunoassays (RIA) supplied by the National Hormone and Pituitary Program of the NIH (Baltimore, MD, USA) as described previously (Loutchanwoot et al., 2008; Roth et al., 2001).

2.6. HPLC analysis for serum levels of test substances

Serum concentrations of EQ and FLUT were analyzed by HPLC-UV detection after extraction and hydrolysis as previously described (Christoffel et al., 2006; Loutchanwoot et al., 2008). In brief, 500 μl of serum were mixed with an equal volume of NH_4 acetate buffer (pH 5.0) containing 1 mg β-glucuronidase (*Helix*