Effects of 8-prenylnaringenin on the hypothalamo-pituitary-uterine axis in rats after 3-month treatment

J Christoffel, G Rimoldi and W Wuttke

Division of Clinical and Experimental Endocrinology, Department of Obstetrics and Gynecology, University of Göttingen, Robert-Koch-Str 40, 37099 Göttingen, Germany

(Requests for offprints should be addressed to W Wuttke; Email: ufkendo@med.uni-goettingen.de)

Abstract

Phytoestrogens are increasingly consumed in artificially high doses as herbal preparations and nutritional supplements. The flavanone 8-prenylnaringenin (8PN) is a potent phytoestrogen, but its benefits and risks after long-term application are poorly identified. Therefore, we tested two doses of 8PN and 17 β -estradiol-3-benzoate (E2B) (effective doses: 6.8 and 68.4 mg/kg body weight (BW) of 8PN, and 0.17 and 0.7 mg/kg BW of 17 β -estradiol (E2)) and compared their effects on uterine weight, pituitary hormones (LH, FSH and prolactin) and the expression of estrogen-regulated genes and of estrogen receptor (ER) α and ER β in the hypothalamus, pituitary and uterus. Both doses of E2 and the high dose of 8PN suppressed serum LH and FSH, and stimulated serum prolactin levels, uterine weight, and progesterone receptor, insulin-like growth factor I and complement protein C3 mRNA transcripts. In the preoptic and the mediobasal areas of the hypothalamus, all treatments had negligible

effects on ER α and ER β and gonadotropin-releasing hormone (GnRH) receptor gene expression, while ER β and GnRH receptor transcripts in the anterior pituitary were reduced under both E2 doses and the high 8PN dose. The mRNA concentrations of the LH α and β subunits in the pituitary were suppressed by E2 and 8PN. In summary, 8PN had very similar though milder effects than E2 on all tested parameters. Inhibition of climacteric complaints by E2 takes place in the hypothalamus, where it inhibits the overactive GnRH pulse generator. Hence, 8PN may be used to inhibit climacteric symptoms effectively. Human pharmacologic studies will show whether the stimulatory effect on the uterus that was found in the present animal model would require the concomitant administration of progestins to prevent endometrial overstimulation.

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Introduction

The flavanone 8-prenylnaringenin (8PN) was identified as a potent phytoestrogen in a Thai crude drug (Kitaoka *et al.* 1998) and as an estrogenic compound in female hops cones (Milligan *et al.* 1999). 8PN is therefore found in beer, in which hops is used as a flavoring and aromatic agent, although the content varies considerably depending on the hop variety used for brewing. The recently published data on mammary cancer- and arteriosclerosis-stimulating effects associated with long-term hormone replacement therapy of postmenopausal women (Rossouw *et al.* 2002) boosted research to establish alternatives to this treatment. Plant-derived phytoestrogens may be such an alternative, and several compounds, including 8PN, have been shown to have estrogenic effects (Milligan *et al.* 2000, 2002, Zierau *et al.* 2002). In recent years, hops has increasingly appeared as a component of herbal preparations for 'bust enhancement' that are mainly available via the Internet (Fugh-Berman 2003), and 8PN proved to

be the main estrogenic compound of a dietary supplement for oral use (Coldham & Sauer 2001). Consumers of these products will be prone to unlimited use. Biologic research with 8PN started only recently, and it was shown that it binds to both estrogen receptors (ER); ER α and ER β . In receptor-binding studies and in transfected yeast cells, it had the strongest ER α activity detected for a phytoestrogen so far (Bovee *et al.* 2004); the ED₅₀ to displace estradiol-17 β (E2) from ER α and ER β was close to 10⁻⁵ M, and 8PN caused transactivation in a receptor transfected yeast screening assay at nM concentrations (Milligan *et al.* 2000, 2002). *In vitro* it lacked androgenic and progestogenic activities (Milligan *et al.* 2000), but mild antiandrogenic activity was demonstrated (Zierau *et al.* 2003). 8PN also inhibits angiogenesis *in vitro* and *in vivo* (Pepper *et al.* 2004). In studies for its estrogenic activity *in vivo*, a dose of 10 mg/kg body weight (BW)/day given once or for 3 days caused uterotrophic response, and a longer s.c. application of 2 weeks (30 mg/kg per day) had positive effects on bone density measured by

dual energy X-ray absorptiometry and urinary bone turnover markers (Miyamoto *et al.* 1998, Diel *et al.* 2004). In ovariectomized (ovx) mice, 0.5 µg 8PN/g food did not evoke uterotrophic response (Coldham & Sauer 2001), whereas s.c. application of 10 mg/kg per day to ovx rats stimulated uterine weight (Diel *et al.* 2004).

Due to the estrogenic potency of 8PN, safety questions arise concerning unrestricted long-term use of freely accessible herbal preparations, and there is a need to study long-term application to evaluate possible benefits and risks.

Therefore, the model of the ovx rat was chosen, which is a widely used and accepted model to study estrogenic activities. A treatment time of 3 months was selected to determine whether the endocrine regulation of hypothalamo-pituitary-gonadal (HPG) axis is altered under constant oral application conditions, which is the consumer's preferred administration route. The so-called negative feedback effect of E2 on pituitary gonadotropin (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) and the stimulatory effect on prolactin release are exerted in the hypothalamus as well as in the pituitary. Hence, both structures express ER α and ER β . The release of the pituitary gonatotropins is stimulated by hypothalamic gonadotropin-releasing hormone (GnRH), of which the receptors in the hypothalamus and the pituitary may also be modulated by E2 and estrogenic compounds. Two doses of both E2 and 8PN were given to study effects on pituitary hormones and the gene-expression profile of estrogen-regulated genes in hypothalamus, pituitary and uterus.

E2 is known to stimulate uterine weight by increased production of insulin-like growth factor I (IGF-I). Furthermore, it stimulates the expression of the progesterone receptor (PR) and of the complement protein C3, which is the most sensitive marker of estrogenic effects in the uterus (Seidlova-Wuttke *et al.* 2003a). Whether E2 alters gene expression of ER α and ER β in the hypothalamus and pituitary is disputed (Shupnik 1996, Shughrue *et al.* 2002, Petersen *et al.* 2003) and will therefore also be studied and the observed effects of E2 compared with those exerted by 8PN.

Most GnRH neurons in the rat hypothalamus are located in the preoptic area (POA), and their axons terminate at the portal vessels of the median eminence of the mediobasal hypothalamus (MBH) (Gore 2002). The GnRH neurons signal their activity to neighboring neurons in both hypothalamic structures, as GnRH receptors are found in the POA and the MBH (Piva *et al.* 2004).

In the pituitary, LH and FSH are synthesized by the gonadotrophs. These two hormones consist of an unspecific α -subunit and an LH-specific β -subunit, of which the mRNA expression may also be regulated by E2 and other estrogenic compounds. Hence, a number of estrogen-regulated parameters in the hypothalamus, pituitary and uterus may serve to study putative estrogenic effects of 8PN and to compare them with those of E2.

Assuming a bioavailability of 10%, which was reported after oral application of isoflavones to rats (Mallis *et al.* 2003), we chose two doses of 8PN to study the effects in the hypothalamo-pituitary-uterine axis and to compare them with a low and high dose of E2. E2 was administered as 17 β -estradiol-3-benzoate (E2B) to improve the low oral bioavailability of E2 (Schleicher *et al.* 1998), and doses were chosen according to experience and standard protocol in the laboratory (Seidlova-Wuttke *et al.* 2003b, 2004a).

Materials and Methods

Chemicals

8PN was kindly supplied by Schering AG, Berlin, Germany. The synthetic product was a racemate of both naturally occurring enantiomers 2S(-)8PN and 2R(+)-8PN (>99% purity certified by NMR). E2B (98.5% purity) was purchased from Sigma.

Animals

Experiments were performed with rats bred in our own animal facilities. Sprague-Dawley rats for the parental generation were obtained from Fa. Winkelmann (Borchen, Germany) and fed soy-free food for at least 3 weeks before they were mated. Their pups were raised and kept under soy-free conditions, and the female offspring entered the experiment. The animals were housed at 23 °C and 55% relative humidity in a 12-h light:dark cycle, and were allowed free access to chow and water. With legal approval (permission no. 509-42502/01-36-03, district authorities of Braunschweig), the experiments were performed according to the German animal welfare regulations.

Study design

Animals were ovx at the age of 4 months. Treatment started immediately afterward by switching them to the 8PN- or E2B-supplemented chow ($n=10-12$) and lasted 3 months. Substances were given orally to imitate the regular route of human ingestion, including resorption restrictions, influence of intestinal flora and first-pass metabolism in the liver. The chow was provided by Ssniff Special Diet, Soest, Germany. Regular diet was the soy-free formulation (Ssniff SM R/M, 10 mm), in which soy proteins are replaced by potato proteins. The supplemented chow was prepared by mixing the test substances with this formulation to homogeneity before the process of pelleting. Concentrations of the two doses of E2B in the chow were 4.3 and 17.3 µg/g, and of 8PN 0.126 and 1.26 mg/g food respectively. All batches were prepared 1 week prior to the start of the experiment. During treatment, the development of body weight (BW), and

food and water intake were surveyed. On the basis of ingested food, it was calculated after termination of the experiments that the animals received daily low and high doses of 8PN of 6.8 and 68.4 mg/kg BW, and of E2B of 0.17 and 0.7 mg/kg BW (values calculated as free E2).

At the end of treatment, animals were killed by decapitation under light CO₂ anesthesia. Trunk blood was collected and wet weight of uterus was determined. Besides the uterus, a number of organs, including brain and pituitary, were obtained. Organ specimens were immediately frozen in liquid nitrogen and stored at -70 °C.

Serum analysis

The blood samples were centrifuged (3000 g, 20 min) and the serum stored at -20 °C for further analysis. LH, FSH, and prolactin were measured by specific RIA supplied by the National Hormone and Pituitary Program of the NIH (Dr A F Parlow, Harbor General Hospital, Torrance, CA, USA), as described previously (Roth *et al.* 2001b). Serum E2 was assayed with a commercially available kit (Estradiol 3rd Generation; DSL, Sinsheim, Germany). To recover 8PN from serum in detectable amounts for HPLC-UV, enzymatic hydrolysis of potential metabolites was performed before serum extraction. A volume of 500 µl serum was extended with 500 µl NH acetate buffer (pH 5.0) containing 1 mg β-glucuronidase (Helix Pomatia β-Glucuronidase Type H1; Sigma) and incubated overnight at 37 °C. The Strata X solid-phase extraction method with a polymeric sorbent was used. Columns of a 60 mg bed mass/3 ml washing and elution volume (8B-S100-UBJ, Phenomenex) were used according to the standard protocol. Methanol was substituted for ethanol, since EtOH was used for food analysis.

The eluted volume was evaporated to dryness in a Speedvac, 4-methyl-umbelliferone (4 MU) serving as an internal STD of the following reconstitution. Analytes were reconstituted with 100 µl EtOH 100%, rinsing the interior of the glass tube and vortexing well. Then the samples were transferred to HPLC vials with 300 µl microinserts, using a 1 ml syringe and filtering through a PVDF membrane 0.45 µm/4 mm filter to remove remaining protein pollutants. An injection volume of 20 µl was chromatographed over a NC 2504.6 mm Hypersil-ODS 5.0 µm column (Bischoff, Leonberg, Germany), the retention time for 8PN being 13.1 min. Total serum levels of 8PN after hydrolyzation were calculated on the basis of a standard curve utilizing serum spiked with 8PN.

RT-PCR

Gene expression was determined by RT-PCR: GnRH in punches of the POA of the hypothalamus; GnRH receptor in the MBH; gonadotropin α- and β-subunits in pituitary;

IGF-I, C3 and PR in uterus; and ERα and ERβ in all except POA.

Extraction of total RNA from the organs or micro-punches of the POA of the hypothalamus and the protocol of the real-time PCR were described in detail previously (Roth *et al.* 2001a, Seidlova-Wuttke *et al.* 2003c). The reactions were run on an ABI Prism 7700 Sequence Detection System (TaqMan; PE Applied Biosystems, Foster City, CA, USA). The primers and probes were synthesized according to published validated systems: ERα and ERβ, IGF-I, C3 and PR (Seidlova-Wuttke *et al.* 2003c); GnRH and its receptor (Roth *et al.* 2001b). Oligonucleotides were purchased from Eurogentec (Seraing, Belgium).

Additional TaqMan-systems (FAM=6-carboxy-fluorescein, TAMRA=6-carboxy-tetramethyl-rhodamine) were as follows:

Rat LH α-subunit=gonadotropin α-subunit (Godine *et al.* 1982)

Forward primer: 5'-TCTTGGACCTTGCGGGA GT-3'

Reverse primer: 5'-GGTGCCCCATCTATCAG TG-3'

TaqMan-Probe: 5'-FAM-TGCCCTGGAGAAGCAAC AGCCCAT-TAMRA3'

Rat LH β-subunit=gonadotropin β-subunit (Chin *et al.* 1983)

Forward primer: 5'-ACCTTCACCACCAGCATCT GT-3'

Reverse primer: 5'-AGCTCACGGTAGGTGCACA CT-3'

TaqMan-Probe: 5'-FAM-CTGCCTTGCTCCCGT GCCTCA-TAMRA3'

Statistics

In the present study, *relative* changes of mRNA levels were analyzed by comparison of the control and treatment groups. Based on the C_t values of the PCR reactions measured in the control group, an average expression level of the analyzed target gene was calculated from the standard curve, which was set at 100%. Individual C_t values in the treatment groups were transformed in relation to this average value of the control group.

Data are presented as means ± S.E.M. Significant differences between control and treatment groups were analyzed by multiple *t*-test comparison (Prism; GraphPad, San Diego, CA, USA). *P* values of <0.05 were considered significant.

Results

Serum E2 levels in ovx control animals were mostly undetectable, while the two E2B doses gave serum

Figure 1 Effects of E2 and 8PN (both low and high dose) on body weight (a) and wet weight of uteri (b). Note the significant estrogenic effect of the high dose of 8PN. * $P < 0.05$ vs control. $n = 10-12$.

concentrations of 149.04 ± 20.61 and 566.84 ± 55.32 pM respectively. Concentrations of 8PN after hydrolyzation of conjugates in the serum of the animals treated with the low or high dose were 14.46 ± 1.95 and 109.80 ± 23.79 μ M respectively.

Body and uterine weights of the animals are depicted in Fig. 1. The treatment with both doses of E2B and the higher dose of 8PN resulted in significantly reduced body weight (Fig. 1a), whereas uterine wet weight in the two E2B groups was significantly higher than in ovx animals, and the high dose of 8PN stimulated uterine weight significantly (Fig. 1b). However, the 8PN low dose had no effect on uterine weight. The effects of the treatments

on uterine mRNA transcripts of ER α (Fig. 2a), ER β (Fig. 2b), PR (Fig. 2c), IGF-I (Fig. 2d) and C3 (Fig. 2e) indicate an effect of 8PN at the high dose which was comparable to the effects exerted by the low dose of E2B.

Serum LH levels were significantly reduced by E2B and the higher dose of 8PN (Fig. 3a). The suppressive effects of E2B and 8PN on serum FSH achieved significance only for the higher E2B dose (Fig. 3b). Serum prolactin levels were significantly increased by the high dose of E2B and 8PN (Fig. 3c). This negative feedback effect of E2B and 8PN on the pituitary gonadotropins was reflected also in the expression of the LH α - and β -subunits and of the GnRH receptor mRNA transcripts, which were significantly reduced under both doses of E2B and the higher dose of 8PN (Fig. 4a, b and e). While ER α transcripts (Fig. 4c) in the pituitary were not affected by E2B or 8PN, both compounds at the higher dose reduced ER β mRNA transcripts significantly (Fig. 4d).

In the POA and MBH, E2B and 8PN had no significant effect on ER α and ER β mRNA transcripts (Table 1). GnRH gene expression in the POA and GnRH receptor transcripts in the MBH also remained, statistically, unaffected by all treatments, although in the POA the amount of ER α was doubled and in GnRH mRNA transcripts it was tripled.

Discussion

The negative feedback of E2 involves neurotransmitters which dampen the activity of the hypothalamic GnRH pulse generator and thereby serum LH levels. After ovx or in postmenopausal women, the lack of estrogen causes changed release of these neurotransmitters (Jarry *et al.* 1986, 1990) and thereby overactivity of the pulse generator. The neurotransmitters involved in generating GnRH pulses spill over into neighboring neurons that regulate temperature and heart beat rate (Tataryn *et al.* 1979, Gambone *et al.* 1984), and this induces hot flushes and the associated tachycardiac attacks. Therefore, LH suppression caused by estrogenic substances alleviates these complaints. High but not low doses of 8PN reduced LH in the long-term treatment as strongly as the low dose of E2B. The ability of 8PN in a high dose to diminish LH release after 3-month treatment suggests that long-term intake could improve hot flushes in menopausal women. On the other hand, the high dose of 8PN increased uterine weight after long-term exposure, although much less than E2B. Uterotrophic effects are undesirable since they stimulate endometrial hyperplasia and the risk of development of endometrial cancer (Feeley & Wells 2001). Therefore, any treatment of postmenopausal women with estrogen at doses that stimulate endometrial proliferation must be accompanied by interval or continuous treatment with progestin, which reduces the cancer risk (Lethaby *et al.* 2004). At the low dose, 8PN was devoid of

Figure 2 Effects of the two doses of E2 and 8PN on uterine ER α (a), ER β (b), PR (c), IGF-I (d) and complement protein C3 (e) gene expression. Note significant effects of 8PN at the high dose on ER β , PR and C3, which are similar to those exerted by E2. * $P < 0.05$ vs control.

uterotropic effects, as demonstrated by the unaffected weights in comparison to the ovx controls. Serum LH levels, however, were also unchanged and therefore – to extrapolate from the animal model – a beneficial effect on hot flushes appears unlikely.

The availability of highly sensitive molecular tools allows us to study more subtle effects. In an attempt to unravel more discrete effects of 8PN in the hypothalamus, pituitary and uterus, we studied expression of several

genes known to be regulated by estrogens. The effects of E2 in the uterus are mediated by the ER α , since ER α , but not ER β , knockout mice have no uterine response to estrogen (Couse & Korach 1999). ER α was affected neither by E2B nor by 8PN, whereas ER β gene expression was reduced by both doses of E2B and by the higher dose of 8PN. Currently, the role of ER β in the uterus is unknown, as ER β -specific compounds are devoid of uterotrophic effects (Seidlova-Wuttke *et al.* 2003a, Hillisch

Figure 3 Effects of E2 and 8PN at two doses on serum LH (a), FSH (b) and prolactin (c). 8PN at the high dose mimicked the effects of E2. * $P < 0.05$ vs control.

Figure 4 Effects of E2 and 8PN of two doses on the gene expression of pituitary LH α - and β -subunits (a and b), ER α and ER β (c and d) and GnRH receptor (e). Note the significant effects of the high dose of 8PN and the LH subunits, ER β and GnRH receptor, which were also seen in the E2-treated animals. * P <0.05 vs control.

et al. 2004); hence, the effects of its downregulation by E2 and 8PN remain enigmatic. The upregulation of IGF-I by E2 and 8PN is a typical estrogenic effect, as IGF-I causes estrogen-induced proliferation of uterine tissue and prepares the endometrium for possible pregnancy (Fazleabas *et al.* 2004), and this response is specified by the high progesterone levels after ovulation (Fazleabas & Strakova 2002). Therefore, increased PR expression is also necessary for maintenance of early pregnancy. It is known that the complement protein C3 is massively upregulated under estrogenic stimuli (Seidlova-Wuttke *et al.* 2004b), and this involves primarily an ER α -mediated mechanism (Harris *et al.* 2002, Seidlova-Wuttke *et al.* 2003b). It was

therefore not surprising to observe a strong upregulating effect of 8PN on C3 gene expression as well. Both doses of E2B and of 8PN were without effect on gene expression of GnRH in the POA, where most perikarya of these neurons are located in the rat (Gore 2002). Moreover, GnRH receptor gene expression in the MBH, where these receptors are expressed (Piva *et al.* 2004), remained unchanged under any of the treatments. This indicates that the LH- and FSH-suppressive effects of E2B and 8PN do not result in changed GnRH production and receptor sensitivity within the hypothalamus, where GnRH is known to exert an ‘ultrashort’ loop feedback on its own secretion (Piva *et al.* 2004). Hence, the hypothalamic

Table 1 Effect of 2 doses of E2 (0.17 and 0.7 mg/animal per day) and 8PN (6.8 and 68.4 mg/animal per day) on mRNA of ER α , ER β , GnRH and GnRH-receptor, given as % of respective control (means $\pm$ S.E.M.)

mRNA transcript		E2 low	E2 high	8PN low	8PN high
Structure POA	ER α	213.9 $\pm$ 62.49	147.6 $\pm$ 28.51	158.1 $\pm$ 38.48	157.8 $\pm$ 34.58
	ER β	164.2 $\pm$ 39.38	121.5 $\pm$ 16.77	120.7 $\pm$ 22.48	111.4 $\pm$ 16.62
	GnRH	284.8 $\pm$ 176.8	294.1 $\pm$ 159.2	116.2 $\pm$ 29.6	52.5 $\pm$ 14.65
MBH	ER α	116.2 $\pm$ 39.67	87.9 $\pm$ 18.45	116.2 $\pm$ 16.29	111.0 $\pm$ 12.22
	ER β	83.3 $\pm$ 15.26	84.8 $\pm$ 18.08	81.0 $\pm$ 7.98	106.0 $\pm$ 8.23
	GnRH-R	591.1 $\pm$ 327.8	70.57 $\pm$ 18.68	156.2 $\pm$ 65.06	122.4 $\pm$ 38.4

mechanisms involved in the negative feedback of estrogens, which result in reduced serum LH levels, are due to other, not yet fully explored hypothalamic mechanisms.

The negative feedback effect of E2B on LH and FSH also involves a pituitary component which is in part shared by 8PN. Both substances reduce ER α , ER β and GnRH receptor mRNA transcripts. Hence, synthesis of bioactive LH and FSH, as well as of the GnRH receptor, appears to be inhibited. Opposite effects were observed earlier under short-term application conditions of E2 (Quinones-Jenab *et al.* 1996, Shupnik 1996). Direct pituitary effects of 8PN were never reported, but, as in the uterus, this flavanone appears to act weakly, like E2, in the pituitary.

E2B significantly increased serum prolactin levels, and this effect was only marginally shared by the high dose of 8PN. This is surprising, as 8PN shared the inhibitory effects of E2 on gonadotropin release. Possibly higher estrogenic power is needed to stimulate lactotrophs than is necessary to inhibit gonadotrophs.

In view of its relevance to human physiology, the serum concentration of E2 and 8PN deserve discussion. Beer, particularly strong ale, but also preparations marketed for bust enlargement, contain relatively high amounts of 8PN. Values of 4 mg/l beer and an identical amount of isoxanthohumol, which can be converted to 8PN by the intestinal flora, have been reported (Stevens *et al.* 1999, Possemiers *et al.* 2005). Hence, intake of high quantities (mg) of 8PN is possible. Pharmacologists claim that doses per kg/BW applied to rats must be 10–15-fold higher than in man in order to exert equipotent effects (Krasovskii 1976, Davidson *et al.* 1986, Vocci & Farber 1988, Schneider *et al.* 2004). Hence, the dose used in the present experiments may well be of importance for human physiology and – in agreement with earlier reports (Milligan *et al.* 2000) – may have adverse effects in the uterus that require addition of a progestin to prevent endometrial hyperplasia and carcinoma (Lethaby *et al.* 2004). The low dose of E2 used in the present experiment can be considered physiologic for rats, as they are in the range seen in proestrus (Smith *et al.* 1975), whereas the higher dose is clearly supraphysiologic.

Altogether, the effects of E2B and 8PN in the hypothalamo/pituitary axis appear to be very similar, and therefore it is highly likely that 8PN will be effective, although less potent, in reducing climacteric complaints, particularly hot flushes, of which the occurrence is closely coupled to the activity of the GnRH pulse generator. As in the hypothalamo/pituitary axis, 8PN at the low dose had no effect on uterine weight, PR, IGF-I or C3 mRNA transcripts. At the high dose, however, these parameters were stimulated, and only at these doses was 8PN inhibitory in the hypothalamo/pituitary axis. Therefore, the ovx rat model suggests that 8PN at doses that affect the GnRH pulse generator would also be effective in the uterus. If this is confirmed in menopausal women, the compound can probably not be used in hormone replacement therapy

without counteracting progestins in any therapeutic scheme.

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