Effects of long-term treatment with 8-prenylnaringenin and oral estradiol on the GH–IGF-1 axis and lipid metabolism in rats

Martina Böttner1,*, Julie Christoffel* and Wolfgang Wuttke

Department of Clinical and Experimental Endocrinology, University of Göttingen, Robert-Koch-Strasse 40, D-37099 Göttingen, Germany
1Department of Anatomy, University of Kiel, Otto-Hahn-Platz 8, D-24118 Kiel, Germany

Abstract

After the heart and estrogen/progestin replacement study and the women’s health initiative study, the prospect of hormone replacement therapy (HRT) on cardiovascular diseases (CVD) has changed dramatically. These findings led to various attempts to search for alternatives for classical HRT, e.g. phytoestrogens. The flavanone 8-prenylnaringenin (8-PN) was identified as a phytoestrogen with strong estrogen receptor-α activity. As the pituitary and the liver are targets for estrogen action, we assessed the effect of ovariectomy (OVX) and long-term treatment (3 months) with 17-β estradiol benzoate (E2B) and 8-PN on pituitary and liver functions in adult OVX rats. Tested doses were 6.8 and 68.4 mg/kg body weight (BW) of 8-PN and 0.17 and 0.7 mg/kg BW of E2B. Our results demonstrate that 8-PN and E2B decreased BW and increased uterus weight. The high doses of E2B and 8-PN increased serum GH and decreased serum IGF-1 levels. E2B dose dependently decreased cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) concentrations in OVX rats. The high dose of 8-PN showed an estrogenic activity regarding cholesterol and LDL regulation but had no effect on HDL concentrations. By contrast, the low dose of 8-PN augmented HDL levels compared with intact rats. Triglyceride levels were raised in response to the high E2B dose but unaffected by 8-PN treatment. Taken together, 8-PN displays an anti-atherosclerotic profile that appears to be even more beneficial than the one displayed by E2B, and thus might demonstrate a remarkable potential for the prevention of CVD associated with estrogen deficiency.


Introduction

Cardiovascular diseases (CVD) are the leading cause of death in women. The metabolic syndrome is a constellation of disorders that might accumulate to become risk factors for CVD (2002). Although this condition may be positively affected by hormone replacement therapy (HRT), the prospect of HRT on CVD has changed dramatically during the past years. The heart and estrogen/progestin replacement study demonstrated an increase of cardiovascular events in the first year after randomization (Hulley et al. 1998). Similarly, recent results from the women’s health initiative study showed an increase in CVD events (Rossouw et al. 2002). These findings led to various attempts to search for alternatives of classical HRT and plant-derived phytoestrogens might be the candidates.

Phytoestrogens are compounds found in plants and fungi that exhibit estrogenic activity both in vivo and in vitro. Some display selective estrogen receptor (ER) modulating activity and could therefore play a beneficial role in the prevention of osteoporosis, menopausal complaints, and CVD. The flavanone 8-prenylnaringenin (8-PN) was identified as the major estrogenic compound in hop (Milligan et al. 1999) and hop-containing dietary supplements are marketed for breast enhancement and advertised to reduce menopausal complaints (Coldham & Sauer 2001). In receptor-binding studies and in transfected yeast cells, 8-PN had the strongest ERα activity detected for a phytoestrogen so far (Bovee et al. 2004).

Data derived from animal models suggest that 8-PN might exhibit several biological activities. It prevented bone loss in ovarioectomized (OVX) rats (Miyamoto et al. 1998) and a recent study from our laboratory indicated that treatment of OVX rats with 8-PN for 3 months showed a reduction of serum luteinizing hormone (LH) and follicle-stimulating hormone indicating that 8-PN might alleviate menopausal discomforts (Christoffel et al. 2006). These data were confirmed by human studies. It could be demonstrated that daily intake over 12 weeks of a hop extract, standardized on 250 µg 8-PN, exerted favorable effects on vasomotor symptoms and other menopausal discomforts (Heyerick et al. 2006). Consistent with these findings, Rad et al. (2006) reported that a single dose of 750 mg 8-PN reduced LH serum levels in postmenopausal women. Interestingly, it appeared from these experiments that 8-PN was well
tolerated by these women. A major drawback, however, was its estrogenicity in the uterus and the mammary gland (Diel et al. 2004, Christoffel et al. 2006, Rimoldi et al. 2006).

Growth hormone (GH) exerts its metabolic actions through a complicated process that is partly dependent and partly independent of insulin-like growth factor-1 (IGF-1). The liver plays a crucial role in the metabolic process. It is a target organ of GH action, the main source of IGF-1, and an estrogen-responsive organ (Leung et al. 2004). Cholesterol is synthesized in the liver and necessary for the synthesis of cell membranes, bile acids, and hormones. Low-density lipoproteins (LDLs) are the principal means by which cholesterol is transported from the liver to peripheral tissues. Oxidized LDL, however, can damage the arterial endothelium and is more likely to accumulate in the arterial intima. High-density lipoprotein (HDL) is mainly responsible for the transport of cholesterol from the periphery to the liver. HDL diminishes endothelial damage and arterial disease risk by promoting removal of excess cholesterol from the body (Goldland 2004). However, the effects of 8-PN on metabolic and lipid parameters have not been determined yet.

To investigate the effect of 8-PN on metabolic parameters, we implemented a long-term paradigm of OVX in adult female rats to mimic estrogen deficiency during menopause. Since ingestion is the physiological route of phytoestrogen consumption in humans, we chose oral application of the test substances. As a bioavailability of 10% was reported after oral application of isoflavones to rats (Mallis et al. 2003), we administered a low and a high dose of 8-PN. A low and a high dose of 17-β estradiol benzoate (E2B) were orally fed as positive controls. After 3 months of treatment, we assessed body and uterus weights, serum levels of GH and IGF-1, triglycerides (TGs), cholesterol, LDL, and HDL.

Materials and Methods

Chemicals

17β-Estradiol benzoate (E2B purity 98-5%) was purchased from Sigma and 8-PN (purity 99-9%) was kindly supplied by Schering AG, Berlin (Germany).

Animals

All experiments were conducted according to the German animal welfare regulations under permission no. 509.42502/01-36.03 district authorities Braunschweig. All experiments conform with the guidelines given in the UFAW Handbook on the Care and Management of Laboratory Animals.

Parental female Sprague–Dawley rats were obtained from Winkelmann (Borchen, Germany) and kept under soy-free food. Offspring were raised under soy-free conditions to eliminate exposure to exogenous estrogenic compounds. Animals were kept under standard conditions: water and food made available ad libitum, lights on from 0600 to 1800 h, room temperature 23 °C, and relative humidity 55%. At the age of 4 months, rats were bilaterally OVX under isoflurane anesthesia. After 3 months of treatment, animals were decapitated under CO2 anesthesia. Uteri were removed and weighed. In order to minimize interindividual variations, blood samples were obtained between 0800 and 1200 h.

Animal diet and batches of test compound

Substances were given orally to imitate the regular route of human ingestion, including resorption restrictions and first-pass metabolism in the liver.

17-β-E2 was administered as its ester E2B to improve the low oral bioavailability of E2. The doses were chosen according to experiences and standard protocol established in the laboratory (Seidlova–Wuttke et al. 2003, Rimoldi et al. 2007). 8-PN doses were chosen according to doses established for other phytoestrogens by our laboratory (Rachon et al. 2007, Rimoldi et al. 2007). The chow was provided by Ssniff special diets GmbH (Soest, Germany). Regular diet was the soy-free formulation Ssniff SM R/M, 10 mm. The supplemented chow was prepared by mixing the test substances with this formulation to homogeneity before the process of pelleting. Concentrations in 1 kg of food were: E2B low 0-0043 g and E2B high 0-0173 g, 8-PN low 0-126 g and 8-PN high 1-26 g/kg food. All batches were prepared 1 week prior to the start of the experiment. Content of 8-PN in the animal chow was determined by HPLC–UV and measured at start, mid-term, and end of the 3-month treatment period to determine stability. Animals were housed as four to six animals per cage. Food consumption was measured twice a week. The amount of food eaten was divided by the number of animals and calculated as food/animal per day. On the basis of ingested food, it was calculated after termination of the experiments that the animals received daily low and high doses of 8-PN of 6-8 and 68-4 mg/kg body weight (BW), and of E2B of 0-17 and 0-7 mg/kg BW (values calculated as free E2).

HPLC analysis

To recover 8-PN from serum in detectable amounts for HPLC–UV, enzymatic hydrolysis of potential metabolites was performed before serum extraction. 500 µl serum were mixed 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. Samples were prepared via the Strata X solid phase extraction and eluted with ethanol. The eluted volume was evaporated till dryness in the speedVac with 4-methyl-umbelliferone (4MU) serving as an internal standard for the following reconstitution. Samples were reconstituted with 100 µl ethanol. Subsequently, samples were filtered through a polyvinylidene difluoride (PVDF) membrane (0-45 µm/4 mm) filter to remove remaining protein pollutants. An injection volume of 20 µl was chromatographed over an NC 250×4-6 mm Hypersil-ODS 5-0 µm column (Bischoff, Leonberg, Germany). Serum spikes
of 4MU, 4MU-glucuronide (4MUG), and 4MU-sulfate (4MUS) were included in each preparation as duplicate controls and detected by the fluorescence of free 4MU (RF-10A XL Fluorescence Detector, Shimadzu, Hanover, Germany): 4MUG and 4MUS served as controls of hydrolysis, 4MU being their reference as well as control of extraction in comparison with the internal standard. The standard curve was obtained from spikes of pooled serum, treated exactly as regular unknowns as described above.

Ethanol, acetonitrile, and water were obtained from J T Baker (Deventer, The Netherlands) and α-phosphoric acid 85% p.a. was supplied by Merck. The analysis of food and serum was performed with the same gradient. The respective extracts were eluted at a flow rate of 1 ml/min with a linear binary gradient of water containing 0-085% α-phosphoric acid (A) and acetonitrile (B): t = 0 min: A 70%, t = 15 min: A 25%, t = 16–21 min: A 0%, t = 22–29 min A 70%. The signal was detected with a u.v./visible spectrophotometer (LC-95, Perkin–Elmer, Waltham, MA, USA) at 220 nm.

**Serum analysis**

The blood samples were centrifuged (3000 g, 20 min) and the serum was stored at −20 °C until further analysis. GH was measured by a specific RIA supplied by the National Hormone and Pituitary Program of the NIH. Briefly, 50 µl serum were added to 200 µl rabbit α-GH antibody (1:5000) and incubated at 4 °C O/N. Subsequently, 100 µl 125I-labeled tracer (20000 c.p.m.) was added and samples were incubated for 24 h at 4 °C. After an addition of the secondary antibody (sheep α-rabbit, 1:30), samples were incubated for 48 h at 4 °C and subsequently centrifuged for 1 h at 2400 g. Radioactivity in the pellets was counted with a Wallac-LKB 1270 and analyzed with the RIA-CALC program. Estradiol levels were assessed using the kit Estradiol 3rd generation (DSL, Sinsheim, Germany): 4MUG and 4MUS served as controls of hydrolysis, controls and detected by the fluorescence of free 4MU (4MUS) were included in each preparation as duplicate controls and detected by the fluorescence of free 4MU (RF-10A XL Fluorescence Detector, Shimadzu, Hanover, Germany): 4MUG and 4MUS served as controls of hydrolysis, 4MU being their reference as well as control of extraction in comparison with the internal standard. The standard curve was obtained from spikes of pooled serum, treated exactly as regular unknowns as described above.

**Statistical evaluation**

Significant differences between the control and treatment groups were analyzed by one-way ANOVA followed by Newman–Keuls post hoc test (PrismTM, GraphPad, San Diego, CA, USA). P < 0.05 were considered significant.

**Results**

We first measured serum levels of estradiol and 8-PN in our animals (Table 1). Intact rats exhibited E2 concentrations of 19.9 pg/ml that dropped upon OVX to 9.5 pg/ml. Animals fed with the low dose of E2B showed serum E2 concentrations of 40.3 pg/ml that lay in the physiological range. Animals fed

### Table 1 Serum levels of 17β-estradiol (E2) and 8-prenylnaringenin (8-PN) in rats. E2 levels were measured via RIA, 8-PN contents were assessed via HPLC. Data are shown as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>E2 (pg/ml)</th>
<th>8-PN (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>19.9 ± 2.2</td>
<td>9.5 ± 1.9</td>
</tr>
<tr>
<td>OVX</td>
<td>40.3 ± 16.7</td>
<td>153.2 ± 49.6</td>
</tr>
<tr>
<td>E2B</td>
<td>153.2 ± 49.6</td>
<td>49.6 ± 3.8</td>
</tr>
<tr>
<td>E2B-h</td>
<td>49.6 ± 3.8</td>
<td>49.6 ± 3.8</td>
</tr>
<tr>
<td>8-PN low</td>
<td>3.8 ± 2.1</td>
<td>3.8 ± 2.1</td>
</tr>
<tr>
<td>8-PN high</td>
<td>3.8 ± 2.1</td>
<td>3.8 ± 2.1</td>
</tr>
</tbody>
</table>

n = 9–12. Since this study is part of a multi-organic risk assessment study, part of these data have been used for other publications (Bottner et al. 2006a,b, Christoffel et al. 2006).

With the high dose of E2B had pharmacological levels of 153.2 pg/ml. Rats fed with the low dose of 8-PN showed serum levels of 14.5 µM 8-PN, rats fed with the high dose exhibited serum concentrations of 109.8 µM.

OVX animals gained 17% of BW compared with intact animals (Table 2). The low and high dose of E2B led to a reduction in BW of 17 or 25% respectively, compared with OVX rats. The low dose of 8-PN led to a reduction of weight gain of 6% and the high dose of 8-PN caused a weight reduction of 20% compared with OVX animals.

To assess estrogenic effects of 8-PN, we measured uterine wet weight. As demonstrated in Fig. 1 both doses of E2B and the high dose of 8-PN increased uterine wet weight compared with OVX and intact animals.

Serum GH levels in intact rats showed 14.4 ng/ml and OVX had no effect. Treatment with the high dose of E2B increased GH levels 2.2-fold, the high dose of 8-PN led to a 1.9-fold increase, both compared with intact control animals (Fig. 2).

OVX had no influence on IGF serum concentrations. IGF-1 levels were decreased by 16% after treatment with the high dose of E2B compared with OVX animals (Fig. 3). Similarly, the high dose of 8-PN reduced serum IGF-1 concentrations by 16% compared with OVX rats. The low dose of 8-PN had no effect on circulating IGF-1.

OVX led to 20% increase in total cholesterol compared with intact animals (Fig. 4).

### Table 2 Body weights. Data are shown as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>291.5 ± 6.0</td>
</tr>
<tr>
<td>OVX</td>
<td>341.1 ± 8.1*</td>
</tr>
<tr>
<td>E2B</td>
<td>284.7 ± 8.9†</td>
</tr>
<tr>
<td>E2B-h</td>
<td>257.2 ± 5.1†</td>
</tr>
<tr>
<td>8-PN low</td>
<td>320.8 ± 6.4†</td>
</tr>
<tr>
<td>8-PN high</td>
<td>271.6 ± 7.1†</td>
</tr>
</tbody>
</table>

n = 9–12. *P < 0.05 versus intact. †P < 0.05 versus OVX.
The low dose of E2B reduced cholesterol to 69% and the high dose to 30% compared with OVX animals. Animals fed with the high dose of 8-PN showed a decrease of the OVX-induced rise in cholesterol to levels found in intact animals whereas the low dose of 8-PN had no effect.

Following OVX, LDL levels were elevated by 66% compared with intact controls (Fig. 5). E2B dose dependently decreased LDL levels to 48 and 8% respectively of OVX animals. The high dose of 8-PN attenuated the OVX-induced increase in LDL. The low dose of 8-PN did not alter the OVX-induced rise in LDL levels.

OVX showed a tendency to elevate HDL levels that, however, did not turn out to be significant (Fig. 6). Treatment with E2 dose dependently decreased serum HDL concentrations to 86 and 12% of intact rats. The low dose of 8-PN led to a 20% increase in HDL levels. HDL concentrations of animals fed with the high dose of 8-PN did not differ from intact animals.

The high dose of E2B caused a 2.1-fold increase in serum TG levels compared with intact control animals (Fig. 7). 8-PN and the low dose of E2B did not alter TG levels.

**Discussion**

This is the first study investigating estrogenic effects of 8-PN on the GH–IGF-1 axis and on lipid profiles in rats. Our results show seven important findings: 1) E2B and the high dose of 8-PN reduce the OVX-induced weight gain; 2) both doses of E2B and the high dose of 8-PN increase uterine wet weight; 3) 8-PN increases serum GH levels and decreases serum IGF-1 concentrations; 4) 8-PN attenuates the OVX-induced increase in total cholesterol; 5) the high dose of 8-PN decreases the elevated LDL levels caused by OVX; 6) the low
dose of 8-PN increases HDL levels compared with intact rats; and 7) TG levels are not affected by 8-PN treatment.

Estrogen agonistic actions of 8-PN are long known. It has been reported that 8-PN displaced E2 from rat uterine cytosol and that 8-PN competed strongly with E2 for binding to ERα and ERβ (Milligan et al. 1999, 2000). Zierau et al. (2005) demonstrated that at a dose of 10⁻⁶ M 8-PN showed stimulation in a yeast-based ERα assay and in an endogenously ERα expressing luciferase assay. At a single dose of 10 mg/day per kg body mass 8-PN stimulated uterine wet weight, increased uterine and vaginal epithelial height, and downregulated ERα and clusterin gene expression in the uterus (Diel et al. 2004). OVX rats treated with 1–30 mg/kg per day 8-PN subcutaneously for 2 weeks showed suppression of bone resorption markers and an increase in bone mineral density (Miyamoto et al. 1998).

Our data reveal that the high dose of 8-PN led to a weight reduction of 20% compared with OVX animals, an effect similar to that observed to the E2B-treated animals. The effect of 8-PN is likely to be mediated via ERα since ERα knockout (KO) mice are obese (Ohlsson et al. 2000). Furthermore, the high dose of 8-PN mimicked the effect of E2B in increasing uterine wet weight. Since these data are part of a multi-organic risk assessment study, these data have been shown in another publication too (Rimoldi et al. 2006) and therefore are discussed there in detail.

Since it has been demonstrated that ERα is expressed in GH-positive cells in the pituitary (Stefaneanu et al. 1994) and as radiolabeled estrogen was found in GH immunoreactive cells (Keefer et al. 1976), we first determined the effect of E2B and 8-PN on pituitary GH secretion. Both the high dose of 8-PN and the high dose of E2B significantly upregulated GH levels. This might be due to a direct effect on the pituitary or regulation might occur on the level of the hypothalamus. Using double-label in situ hybridization, Kamegai et al. (2001) showed that 70% of GHRH neurons contain ERα mRNA indicating that both substances may directly act on GH-releasing hormone (GHRH) neurons through ERα to modify GH secretory patterns. In contrast to the observed upregulation of GH, we noticed a downregulation of circulating IGF-1 by the high doses of E2B and 8-PN. This finding is in agreement with the human studies that also report reductions of IGF-1 serum concentrations following estrogen treatment (Wiedemann et al. 1976, Duursma et al. 1984). Thus, the rise in GH can partly be interpreted as a negative feedback to the fall in IGF-1, which agrees with the negative feedback between IGF-1 and GH, as has been demonstrated by Berelowitz et al. (1981) in cultured rat hypophyseal cells.

As an atherosclerotic lipid profile is a major risk factor for CVD, we next assessed serum levels of lipids and lipoproteins in our animals. E2B dose dependently decreased cholesterol and LDL concentrations. Similarly, the high dose of 8-PN attenuated the OVX-induced rise in cholesterol and LDL.
levels. A recent study showed that E₂ inhibits the first enzyme in cholesterol biosynthesis, thus reducing the synthesis of cholesterol (Moorthy et al. 2004). Furthermore, the effect on cholesterol metabolism might at least partly be mediated by ERα since ERαKO but not ERβKO mice display elevated levels of total cholesterol (Ohlsson et al. 2000). The same study reports that disruption of the ERα gene results in an atherogenic lipoprotein profile characterized by an increase in smaller and presumably denser LDL particles. Hence, it is likely that the effects of 8-PN on lipid metabolism are mediated via ERα, particularly since 8-PN has been shown to be the most potent phytoestrogen with ERα (Bovee et al. 2004). Moreover, estrogen has been demonstrated to upregulate the LDL receptor, leading to the increased clearance of LDL from the serum (Knopp 2002).

Remarkably, we observed a dose-dependent decrease of HDL levels in our E₂B-treated animals whereas the low dose of 8-PN increased HDL concentrations. This finding is in accordance with the previous reports from our group that demonstrate reduced HDL levels following a 3-month treatment with subcutaneously applied E₂ (Bottner & Wuttke 2006) or following a 5-day treatment with E₂ valerate that was applied per gavage (Klammer et al. 2005). By contrast, the 8-PN-induced increase of HDL serum concentrations is in accordance with the results from human studies that consistently demonstrate an increase in HDL levels following estrogen treatment (Friday et al. 2001, Fait et al. 2002, Saglam et al. 2002).

Treatment with 8-PN showed no effect on TG levels whereas the supraphysiologic dose of E₂B increased TG concentrations significantly. This effect might be due to the actions of E₂ on hepatic TG lipase since it has been demonstrated that ethynylestradiol administration downregulates the activity of this enzyme by decreasing its mRNA levels (Staels et al. 1990). In agreement with this finding, further studies report the inhibition of hepatic lipase by E₂ (Applebaum-Bowden et al. 1989, Kushwaha et al. 1990).

Our data reveal that 8-PN acts as an estrogen agonist on GH and IGF–1 secretions as well as on cholesterol and LDL serum levels. These actions are likely to be mediated mainly by ERα, which is in accordance with the findings that characterized 8-PN as an ERα agonist. Using a mamalian cell-based transactivation assay, Schaefer et al. (2003) demonstrated that 8-PN is the strongest plant-derived ERα agonist identified so far. These results were confirmed by Bovee et al. (2004) who showed in a yeast transcription activation assay stably expressing human ERα and ERβ that 8-PN is more potent with ERα than with ERβ.

The dose of 8-PN to elicit maximal estrogenic effects (68.4 mg/kg BW) was 97 times higher than the high dose of E₂B (0.7 mg/kg BW). This is due to the fact that the doses of phytoestrogens applied to elicit estrogenic effects are generally higher than those required for steroids as demonstrated by numerous studies including those for 8-PN. Dietl et al. (2004) showed that an injection of a dose of 10 mg/kg per day of 8-PN stimulated uterine wet weight and increased uterine epithelial height to a similar degree as observed with a dose of 0.03 mg/kg per day of E₂. Similarly, Humpel et al. (2005) demonstrated that the 8-PN-mediated prevention of bone loss required an injection of 20 mg/kg per day while E₂ was effective at a dose of 0.004 mg/kg per day. The main reason for the observed differences is the fact that the estrogenic potency of 8-PN is at least 100 times lower than that of E₂. In a rapid yeast estrogen bioassay, Bovee et al. (2004) reported that the relative estrogenic potency of 8-PN was 0.01 with ERα and 0.0039 with ERβ.

Taken together, 8-PN displays an anti-atherosclerotic profile that appears to be even more beneficial than the one displayed by E₂B. 8-PN treatment did not decrease HDL levels nor increase TG serum concentrations. Thus, 8-PN displays a remarkable potential for the prevention of CVD associated with estrogen deficiency.

Declaration of Interest

There is no conflict of interest that would prejudice the impartiality of the research reported.

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