

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

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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.

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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 ER α 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 ovariectomized (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 μ g 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 (Godsland 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

17 β -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 μ l serum were mixed with 500 μ l 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 μ l ethanol. Subsequently, samples were filtered through a polyvinylidene difluoride (PVDF) membrane (0.45 μ m/4 mm) filter to remove remaining protein pollutants. An injection volume of 20 μ l was chromatographed over an NC 250 \times 4.6 mm Hypersil-ODS 5.0 μ m 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 *o*-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% *o*-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 ^{125}J -labeled tracer (20,000 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) according to the manufacturer's guidelines. IGF-1 levels were assessed via RIA according to the manufacturer's guidelines (DSL). Cholesterol, LDL, HDL, TG, and glucose were measured with a Hitachi 902 (Boehringer Mannheim).

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 E_2 concentrations of 19.9 pg/ml that dropped upon OVX to 9.5 pg/ml. Animals fed with the low dose of $E_2\text{B}$ showed serum E_2 concentrations of 40.3 pg/ml that lay in the physiological range. Animals fed

Table 1 Serum levels of 17 β -estradiol (E_2) and 8-prenylningenin (8-PN) in rats. E_2 levels were measured via RIA, 8-PN contents were assessed via HPLC. Data are shown as mean \pm S.E.M.

Serum levels	
Treatment group	
Intact	19.9 (± 2.2) pg/ml
OVX	9.5 (± 1.9) pg/ml
$E_2\text{B-l}$	40.3 (± 16.7) pg/ml
$E_2\text{B-h}$	153.2 (± 49.6) pg/ml
8-PN low	4.909 $\mu\text{g/ml}$ (14.46 μM)
8-PN high	37.28 $\mu\text{g/ml}$ (109.8 μM)

$n=9-12$.

Since this study is part of a multi-organic risk assessment study, part of these data have been used for other publications (Böttner *et al.* 2006a,b, Christoffel *et al.* 2006).

with the high dose of $E_2\text{B}$ 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 $E_2\text{B}$ 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 uterus weights. As demonstrated in Fig. 1 both doses of $E_2\text{B}$ 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 $E_2\text{B}$ increased GH levels 2.1-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 $E_2\text{B}$ 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 \pm S.E.M.

Body weights (g)	
Treatment group	
Intact	291.5 (± 6.0)
OVX	341.1 (± 8.1)*
$E_2\text{B-l}$	284.7 (± 8.9) [†]
$E_2\text{B-h}$	257.2 (± 5.1)*, [†]
8-PN low	320.8 (± 6.4)*, [†]
8-PN high	271.6 (± 7.1)*, [†]

$n=9-12$, * $P<0.05$ versus intact, [†] $P<0.05$ versus OVX.

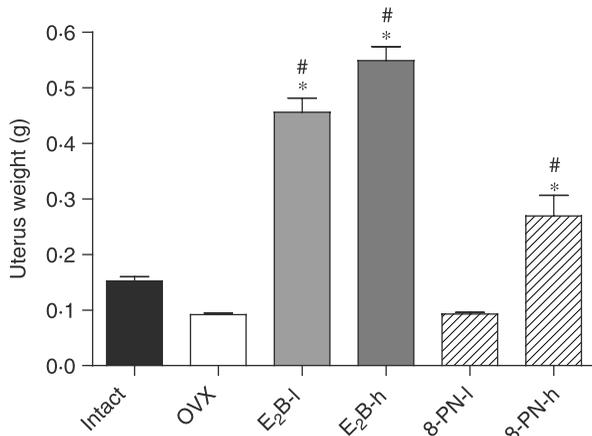


Figure 1 Uterus weights. Data are shown as mean \pm s.e.m., $n=9-12$, * $P<0.05$ versus intact, [#] $P<0.05$ versus OVX.

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.

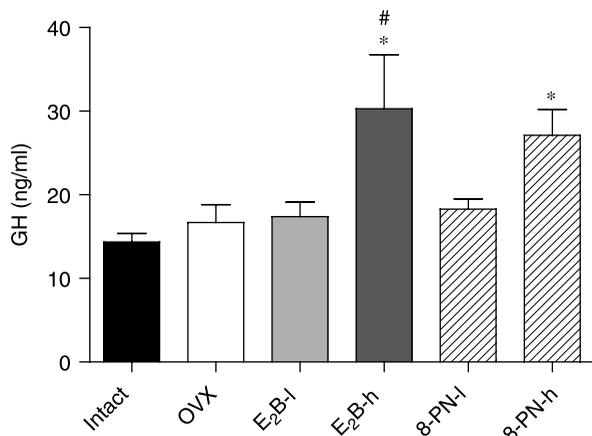


Figure 2 Serum levels of GH. GH levels were measured via RIA. Data are shown as mean \pm s.e.m., $n=9-12$, * $P<0.05$ versus intact, [#] $P<0.05$ versus OVX.

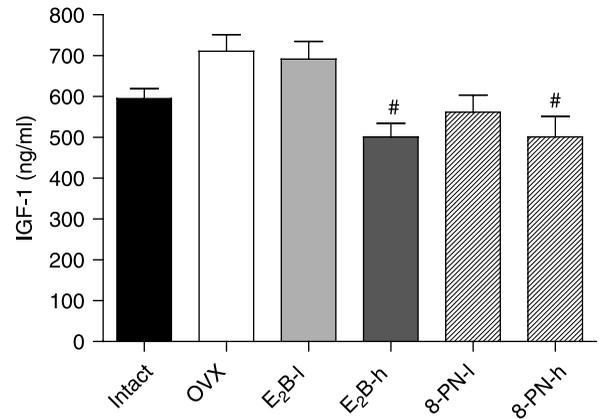


Figure 3 Serum levels of IGF-1. IGF-1 concentrations in serum samples were assessed via RIA. Data are shown as mean \pm s.e.m., $n=9-12$, [#] $P<0.05$ versus OVX.

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

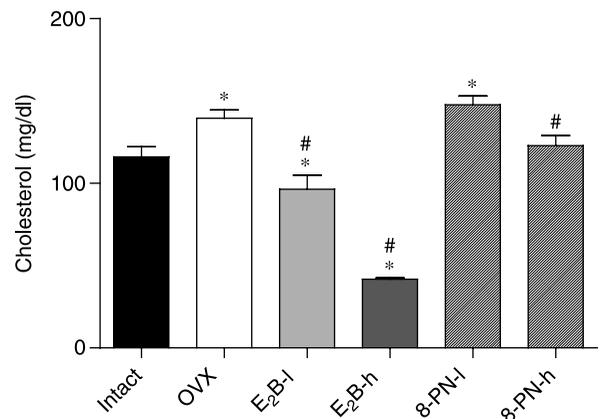


Figure 4 Serum concentrations of total cholesterol. Cholesterol concentrations were assessed with a Hitachi. Data are shown as mean \pm s.e.m., $n=9-12$, * $P<0.05$ versus intact, [#] $P<0.05$ versus OVX.

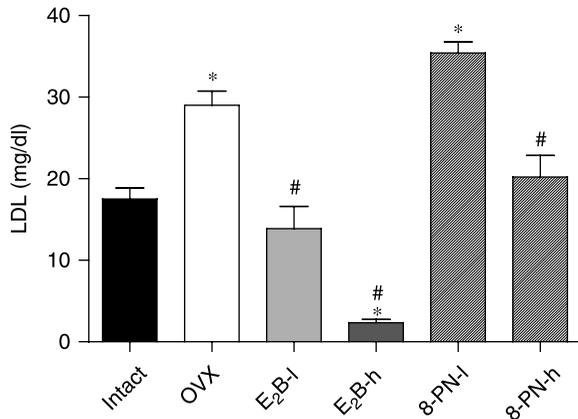


Figure 5 Serum concentrations of LDL. LDL concentrations were measured with a Hitachi. Data are shown as mean \pm S.E.M., $n=9-12$, * $P<0.05$ versus intact, # $P<0.05$ versus OVX.

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 ER α and ER β (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 ER α assay and in an endogenously ER α 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 ER α 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

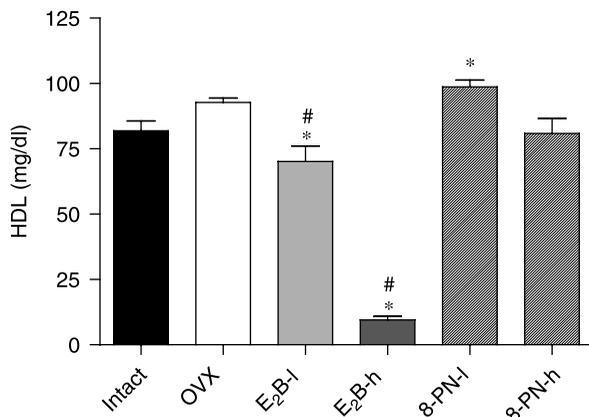


Figure 6 Serum concentrations of HDL. HDL concentrations were measured with a Hitachi. Data are shown as mean \pm S.E.M., $n=9-12$, * $P<0.05$ versus intact, # $P<0.05$ versus OVX.

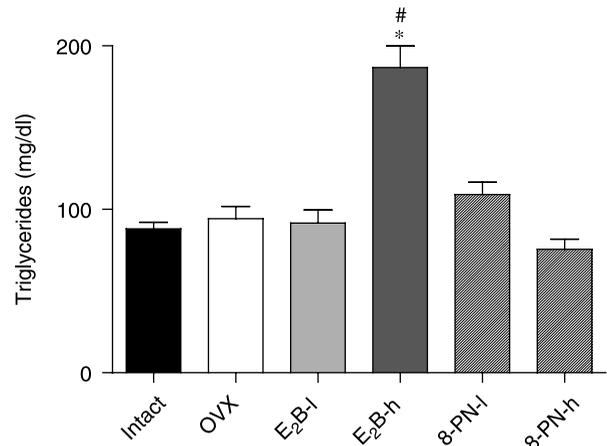


Figure 7 TG serum levels. TG concentrations were determined with a Hitachi. Data are shown as mean \pm S.E.M., $n=9-12$, * $P<0.05$ versus intact, # $P<0.05$ versus OVX.

similar to that observed to the E₂B-treated animals. The effect of 8-PN is likely to be mediated via ER α since ER α 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 ER α 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 ER α mRNA indicating that both substances may directly act on GH-releasing hormone (GHRH) neurons through ER α 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 hypophysal 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 ER α since ER α KO but not ER β KO mice display elevated levels of total cholesterol (Ohlsson *et al.* 2000). The same study reports that disruption of the ER α 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 ER α , particularly since 8-PN has been shown to be the most potent phytoestrogen with ER α (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 (Böttner & 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 ethinylestradiol 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 ER α , which is in accordance with the findings that characterized 8-PN as an ER α agonist. Using a mammalian cell-based transactivation assay, Schaefer *et al.* (2003) demonstrated that 8-PN is the strongest plant-derived ER α agonist identified so far. These results were confirmed by Bovee *et al.* (2004) who showed in a yeast transcription activation assay stably expressing human ER α and ER β that 8-PN is more potent with ER α than with ER β .

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. Diel *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 ER α and 0.0039 with ER β .

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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