17β-Estradiol but not the phytoestrogen naringenin attenuates aortic cholesterol accumulation in WHHL rabbits

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Abstract **The effects of 17β-estradiol (17β-E_2) or the phytoestrogen naringenin on spontaneous atherosclerosis were studied in 36 ovariectomized homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits receiving a semisynthetic control diet; this diet added 0.0040% 17β-E_2, or 0.20% naringenin, for 16 weeks. The uterine weight was increased ($P < 0.001$) and the concentration of estrogen receptor α was decreased ($P < 0.001$) in the 17β-E_2 group compared with the controls. Total plasma cholesterol and triglycerides were not different from those in the controls. In lipoproteins, HDL cholesterol was increased ($P < 0.01$), and LDL triglyceride and IDL triglyceride were lowered ($P < 0.05$). The oxidation (as concentration of malondialdehyde) was increased in LDL ($P < 0.05$) but not in plasma. The cholesterol accumulation was decreased ($P < 0.05$) in the ascending aorta and in the total aorta but the ratio of intima to media and area of intima in ascending, thoracic, and abdominal aorta were not significantly different. In the naringenin group the only differences, compared with the control group, were increased HDL cholesterol ($P < 0.001$) and decreased activity of glutathione reductase ($P < 0.05$). In conclusion, 17β-E_2, but not naringenin, attenuated aortic cholesterol accumulation independently of plasma and LDL cholesterol. Further, these results support previously suggested pro-oxidant ability of 17β-E_2 toward LDL and a possible connection between the pro-oxidant nature of 17β-E_2 and its antiatherogenic effect.**—Mortensen, A., V. Breinholt, T. Dalsgaard, H. Frandsen, S. T. Lauridsen, J. Laigaard, B. Ottesen, and J-J. Larsen. **17β-Estradiol but not the phytoestrogen naringenin attenuates aortic cholesterol accumulation in WHHL rabbits.** ***J. Lipid Res.*** **2001.** 42: **834–843.**

Supplementary key words hormone • lipids • atherosclerosis

Atherosclerotic cardiovascular disease is the most common cause of death in both men and women in Western societies. The fact that the risk of cardiovascular disease is similar in men and postmenopausal women, and that cardiovascular disease is less prevalent in premenopausal women and women receiving estrogen replacement therapy than in postmenopausal women, suggests a protective effect of estrogens. Indeed, antiatherogenic properties of 17β-estradiol (17β-E_2) have been demonstrated in several animal models (1–6). Furthermore, some human data indicate an antiatherogenic effect of 17β-E_2 (7, 8).

Dietary flavonoids, present in a number of edible plants, are currently attracting considerable attention because of their estrogenic and antioxidant properties. They are members of a large flavonoid family that shares with steroidal estrogens the ability to bind to the estrogen receptor and to mediate transcription of estrogen-responsive genes. Flavonoids are believed to be major contributors to the beneficial effect of foods of plant origin on coronary heart disease (CHD). Moreover, epidemiological studies indicate that the intake of dietary flavonoids is significantly inversely associated with mortality from CHD (9–11). Thus, dietary flavonoids with estrogenic properties (so-called phytoestrogens) could possibly be important for postmenopausal women as a natural supplement, or as an alternative to hormone replacement therapy, in prevention of cardiovascular disease. Consequently, a study was designed to evaluate the effect of 17β-E_2 and the phytoestrogen naringenin (**Fig. 1**) on development of spontaneous atherosclerosis in ovariectomized female Watanabe heritable hyperlipidemic (WHHL) rabbits. The flavonone naringenin, naturally occurring in citrus fruits, was chosen because it has a relatively high in vitro estrogenic activity compared with other flavonoids (12) and it counts for about 10% of the total estimated human flavonoid intake in Denmark (13). Furthermore, the results from several in vitro studies indicated its antiatherogenic activities [reviewed in ref. (14)], but no information about the pos-

Abbreviations: CHD, coronary heart disease; 17β-E_2, 17β-estradiol; Erα, estrogen receptor α; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione transferase; MDA, malondialdehyde; SOD, superoxide dismutase; UDPGT, UDP-glucuronosyltransferase; WHHL, Watanabe heritable hyperlipidemic.

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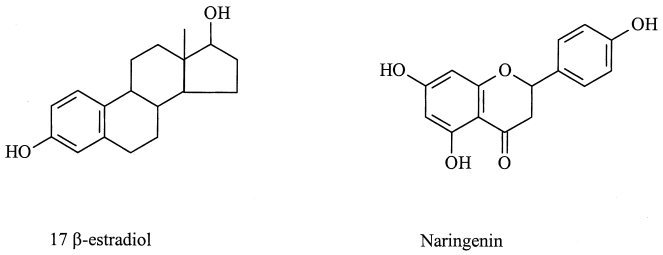

Fig. 1. Chemical structures of the test compounds 17β-E_2 and naringenin.

sible in vivo action of naringenin in animal models of atherosclerosis is available. To mimic more closely human hypercholesterolemia, caused either by genetic or acquired abnormalities in the synthesis or degradation of plasma lipoproteins, which transport endogenous cholesterol between body tissues, the WHHL rabbit was chosen instead of a cholesterol-fed animal model for atherosclerosis. In cholesterol-fed animal models, hypercholesterolemia is induced exogenously, when the normal mechanisms of lipoprotein clearance are overwhelmed by large amounts of dietary cholesterol. In contrast, the WHHL rabbit, fed a standard diet, develops endogenous hypercholesterolemia due to a genetic defect: a deficiency in LDL receptor analogous to the genetic defect in human FH and atherosclerosis with morphological resemblance to the human disease (15, 16). Furthermore, the potential effects of estrogens on hepatic LDL receptors are avoided in this animal model. The atherosclerotic end point in this study was aortic atherosclerosis evaluated by biochemical and microscopy methods. Furthermore, blood lipids were monitored and several biomarkers for redox status such as malondialdehyde (MDA) in plasma and LDL along with various antioxidant enzyme activities in erythrocytes and phase 2 enzymes were included. In addition, estrogen receptors in the uterus were examined.

MATERIALS AND METHODS

Chemicals, test compounds, and diets

Naringenin was purchased from Apin Chemicals (Abington, Oxon, UK). 17β-E_2 was kindly donated by Novo Nordisk Farmaka (Lyngby, Denmark). MDA was purchased from Aldrich (Steinheim, Germany). Butylated hydroxytoluene (BHT), NADPH, 2-thiobarbituric acid (TBA) and EDTA were from Sigma (St. Louis, MO). All other chemicals were from Merck (Darmstadt, Germany). Triglyceride and protein kits were from Roche (Basel, Switzerland). The cholesterol kit was from Boehringer Mannheim (Mannheim, Germany).

Three semisynthetic pelleted diets, C 2000 (g/kg: crude protein 171.10, crude fat 50.52, crude fiber 238.37, ash 84.33, moisture 65.70, disaccharide 184.90, polysaccharide 195.80; metabolizable energy 2,654 kcal/kg), C 2000 with 0.0040% 17β-E_2 added, and C 2000 with 0.20% naringenin added, were obtained from Altromin International (Lage, Germany). Before the study, the semisynthetic diet C 2000 was tested in this laboratory for the specific content of the major isoflavonoids commonly found in soy and soy-based diets. This was done by extraction with water-methanol-formic acid followed by analysis with HPLC, and liquid chromatography-mass spectrometry (LC-MS), and comparing it with a normal rabbit diet based on soybeans. No traces of soy phytoestrogens were found in this diet.

Animals, housing, and clinical observation

Thirty-six homozygous female WHHL rabbits [mean plasma cholesterol, 26.4 ± 4.8 (mean ± SD) mM at 4 weeks of age] were obtained from this breeding colony (derived from a parent generation obtained from F. Jansen, University of Nijmegen, The Netherlands with permission from Y. Watanabe). The animals were housed individually in stainless steel cages under controlled environmental conditions (temperature 18 ± 2°C, relative humidity 55 ± 5%, 12/12-h light/dark cycle, air changed 10 times/h) and observed at least twice a day for any abnormalities in clinical conditions.

Experimental design

At the beginning of the seventh week of life, the rabbits were anesthetized with a 0.40-ml/kg body weight mixture of Hypnorm [fentanyl citrate (0.315 mg/ml) plus fluanisone (10 mg/ml); Janssen-Cilag, Sauderton, High Wycombe, Buckinghamshire, UK], Dormicum (5 mg/ml; F. Hoffman-La Roche, Basel, Switzerland), and sterile water (1 ml plus 1 ml plus 2 ml). A bilateral ovariectomy was then performed. At the start of the eighth week of life the rabbits were randomly distributed into the three experimental groups and each animal received 100 g of diet per day: group I (control) consisted of 12 animals and received C 2000, group II (13 animals) received C 2000 with 0.0040% 17β-E_2 added, and group III (11 animals) received C 2000 with 0.20% of naringenin added. The rabbits were given water ad libitum. The feed intake was recorded daily and body weight was recorded weekly. Blood samples were collected from the marginal ear vein of unanesthetized animals fasted overnight, into tubes containing sodium heparin, before dosing and once monthly thereafter. After 16 weeks of treatment, the rabbits were killed by intravenous injection of pentobarbital (100 mg/kg body weight) into an ear vein. A midline incision was made, the caval vein was transected for exsanguination, and the central arterial system was perfused with about 500 ml of 0.9% NaCl through a cannula inserted into the left ventricle. The lungs, heart, and the entire aorta to 1 cm distal to bifurcation of the iliac arteries, liver, and uterus were removed. The aorta was cleared of adventitial fat. Three cross-sections of the unopened aorta were taken from the ascending aorta just above the aortic valves, from the thoracic aorta at the level of the first intercostal arteries, and from the abdominal aorta just proximal to the celiac artery. All aortic rings were fixed in 4% buffered formalin and were later processed for histological examination. Six to eight serial sections from each sample were stained with elastic hematoxylin-eosin and elastic van Gieson, respectively. The remaining parts of the aorta were opened longitudinally and pinned on a cork board, and the area was outlined on graph paper. These aortic tissues were then divided into the intima-inner media and the outer media, weighed, and stored at −20°C until biochemical analysis. The liver was weighed after removal of the gallbladder and stored at −20°C until biochemical analysis. The mesometrium and fat were removed from the uterus and the uterine horns were detached from the cervix. After removal of lumenal fluid, the entire uterus was wet weighed to assess in vivo exposure to 17β-E_2 and stored at −20°C until biochemical analysis.

Plasma concentration of estradiol

Before euthanasia, a blood sample was collected from the marginal ear vein of seven unanesthetized rabbits fasted overnight and randomly chosen from each group. Plasma was prepared

after centrifugation. The measurement of plasma estradiol was performed at Statens Serum Institut (Copenhagen, Denmark).

Plasma concentration of naringenin

Three untreated and intact WHHL female rabbits fasted overnight and weighing 2.49 ± 0.03 (SD) kg received orally by gavage a single dose of naringenin at 70 mg/kg body weight, in suspension, prepared with 100 ml of distilled water, 10 g of pulverized semisynthetic diet C 2000, and 1.4 g of naringenin. Blood samples were collected before and 2, 6, 12, and 24 h after dosing. The concentration of naringenin in the respective samples was determined by the HPLC method previously described (17).

Automated assays of plasma and lipoproteins

The plasma cholesterol and triglyceride levels were measured before dosing and once monthly thereafter, and their concentrations in lipoproteins were measured at termination. Plasma was isolated from blood samples by centrifugation at 2,000 *g* for 10 min at 4°C. Lipoproteins were separated by a single density gradient ultracentrifugation for 18 h at 21°C (18), using 1 ml of plasma. The density gradient solution contained 0.25 mM EDTA and 0.1 mM BHT. Cholesterol, triglyceride, and protein levels were determined in plasma and lipoproteins, using a Cobas Mira S analyzer (Roche) and relevant kits. Cholesterol and triglyceride concentrations in lipoproteins and plasma were expressed as millimoles per liter (mM) and protein concentrations as grams per liter (g/l).

Determination of MDA

Total MDA was determined in plasma and lipoproteins at termination by an MDA-HPLC method described previously (19). Briefly, the antioxidant BHT was added to the sample to a final concentration of 1.0 mM, and an alkaline hydrolysis was performed by adding NaOH (final concentration, 0.50 M) and heating for 30 min at 60°C to release the protein-bound MDA (20). Samples were reacted with TBA (21) and after a centrifugation at 10,000 *g* for 5 min, the supernatants were analyzed by HPLC: samples (40-μl aliquots) were chromatographed with a linear gradient (0%–50%, 17 min) of H_2O-acetonitrile with 0.1% trifluoroacetic acid. The HPLC analysis was performed with a Hewlett-Packard (Waldbromn, Germany) 1100 system with diode array detector, using a Purospher RP-18 column (4 × 250 mm, 5 μm; Hewlett-Packard) and detection at 532 nm. Four MDA standards were included in each TBA reaction and HPLC run. Sample peaks with retention times and UV spectra identical to the MDA-TBA standards were identified and the concentration of MDA was calculated from a standard curve and expressed as nanomoles of MDA per gram of protein.

Red blood cell antioxidant enzymes

Antioxidant enzyme activities were determined in red blood cell lysates at termination. Heparinized blood samples were centrifuged at 1,500 *g* for 10 min at 4°C and the plasma was removed. The red blood cells were washed three times with 1 volume of 0.9% NaCl, resuspended in 1 volume of deionized water for lysis, and stored at −80°C. Automated assays were performed with a Cobas Mira S analyzer to determine the activity of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase. The activity of the enzymes was related to the amount of hemoglobin in the erythrocyte hemolysates. The SOD and GPx activities and the hemoglobin concentration were determined with commercially available kits. GR activity and catalase activity were determined according to methods previously described (22).

Determination of phase 2 enzymes

The activities of UDP-glucuronosyltransferase (UDPGT) and glutathione transferase (GST) from liver tissue were determined with a Cobas Mira S analyzer according to the methods of Ernster (23), Mulder and van Doorn (24), and Habig, Pabst, and Jacoby (25), respectively. UDPGT and GST activities were measured with 3-methyl-2-nitrophenol and 1-chloro-2,4-dinitrobenzene as substrates, respectively. All samples were analyzed in duplicate.

Preparation of nuclear and cytosolic fractions and determination of estrogen receptor α (ERα)

The uterine tissue was thawed on ice and homogenized in 0.10 M K_3HPO_4/KH_3PO_4, 0.0015 M K_3EDTA, 0.01 M monothioglycerol, 0.01 M sodium molybdate, 10% (v/v) glycerol, pH 7.5, according to Thorpe (26). Before homogenization, the tissue (less than 30 mg) was cut in small pieces (~1 mm^3), 1 ml of ice-cold homogenization buffer was added, and the mixture was homogenized at 1,000 rpm 10 times (10 s each) in a Potter-Elvehjem homogenizer and centrifuged at 800 *g* for 10 min at 4°C. The supernatant (cytosolic fraction) was saved on ice. The pellet was resuspended/washed in another 1 ml of homogenization buffer and centrifuged at 800 *g* for 10 min at 4°C. This procedure was performed twice. The pellet was then resuspended in 1 ml of extraction buffer containing 10 mM Tris-HCl, 0.6 M KCl, 1 mM monothioglycerol, 10% (v/v) glycerol, pH 8.5, and incubated at 4°C for 1 h with vortexing every 15 min. The extracted nuclei and the cytosolic fractions were centrifuged at 105,000 *g* for 45 min at 4°C. The cytosolic and nuclear supernatants were transferred to ice-cold cryotubes and kept at −80°C until analysis. The concentration of estrogen receptor in the cytosolic and nuclear fraction was determined by use of a monoclonal antibody kit (ER-EIA; Abbott, Abbott Park, IL). The receptor content in both cytosol and nuclear fraction was given relative to the protein concentration in the cytosol. The protein concentration was determined by the bicinchoninic acid method (27), adapted to a Cobas Mira S (Roche). Each tissue was measured in duplicate.

Microscopic examination of aortic atherosclerosis

Quantitation of atherosclerotic lesions was performed by point counting. A projective device (Ocular Periplan 12.5/20, Wild Leitz; Leica Microsystems, Heidelberg, Germany) was used to transfer the microscopic image of the aortas to a grid with regularly spaced points (point grid). The degree of magnification and the size of the point grid were kept constant. Quantitation was always performed in two to four serial sections and the mean value was reported. The number of points covering the intima and the number of points covering the media were recorded (16). The quantitative evaluation of atherosclerosis in the ascending, thoracic and abdominal aorta was expressed in two different ways: as the ratio of intima to media and as the area of intima in square millimeters (mm^2).

Biochemical evaluation of aortic atherosclerosis

Weighted aortic intima-inner media from the ascending (from the aortic valves to the first intercostal arteries), thoracic (from the second intercostal arteries to the celiac artery), and abdominal (from the celiac artery to 1 cm distal to the bifurcation of the iliac arteries) aorta was placed in a glass vial with 2 ml of a Triton X-100 (25 g/l)-acetone solution, and the suspension was homogenized three or four times for 15 s with an Ultra-Turrax homogenizer until the aorta was completely pulverized. The sample was centrifuged for 10 min at 4,000 *g* and the supernatant was removed. The cholesterol concentration in the supernatant was determined by a color reaction, using a cholesterol kit (Boehringer Mannheim) and a serum standard. The absorption was read at 500 nm on a Shimadzu (Kyoto, Japan) spectropho-

TABLE 1. Body weight, feed intake, and dose of test compounds of ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

Group/Diet	No. of Animals	Body Weight		Relative Feed Intake	Dose of Test Compound
		Initial	At Termination		
		kg		*g/kg body weight/day*	*mg/kg body weight/day*
I. Control	12	1.17 ± 0.20	2.43 ± 0.42	36.8 ± 4.6	—
II. 0.004% 17β-E_2	13	1.25 ± 0.24	2.33 ± 0.60	30.5 ± 5.6[a]	1.2 ± 0.2
III. 0.2% naringenin	11	1.27 ± 0.19	2.66 ± 0.63	36.1 ± 3.2	72.2 ± 6.4

[a] $P < 0.01$, compared with the control group, Student's *t*-test.

tometer and the concentration was calculated from a standard curve. The results were expressed as nanomoles of total cholesterol per square centimeter of aorta.

Statistics

All results are expressed in terms of means ± SD. The range of data and the median are, in addition, shown for morphological parameters. All data were tested for normal distribution. The data on total plasma cholesterol and triglycerides were compared by repeated measure analysis. All other normally distributed data were compared by Student's *t*-test. Any not normally distributed data were transformed. If normal distribution was achieved, the data were compared by Student's *t*-test; if not, the data were analyzed by the Wilcoxon test. Correlations between cholesterol content in the total aorta and the time-averaged plasma total cholesterol for individual treatment groups were calculated by the Pearson correlation. The effects were considered significant for $P < 0.05$. All analyses were performed with Statistical Analysis System (SAS) software (release 6.12, 1996; SAS Institute, Cary, NC).

RESULTS

Systemic effects of 17β-E_2 and naringenin

As seen in **Table 1**, the initial and terminal body weights were similar in the three groups. The relative feed intake in the 17β-E_2 group was significantly lower than, and in the naringenin group comparable to, that in the control group. No effect of the treatment on clinical appearance was observed in any of the rabbits.

The measurement of estradiol in plasma from seven randomly chosen rabbits from each experimental group demonstrated the following: in the control group, three rabbits had estradiol concentrations under the detection limit of 40 pM, one was at the detection limit, two had an estradiol concentration of 41 pM, and one had an estradiol concentration of 53 pM; in the 17β-E_2 group, the average (±SD) concentration of estradiol was 129 ± 21 pM; in the naringenin group, five rabbits had estradiol concentrations under the detection limit, one had a concentration of 59 pM, and one had a concentration of 75 pM.

The bioavailability of naringenin was measured in intact female WHHL rabbits as the plasma concentration after a single oral dose of 70 mg/kg body weight. The concentration after 2 h was 0.73 ± 0.37 μg/ml. The peak was recorded 6 h after dosing, reaching 1.34 ± 0.81 μg/ml. The concentration after 12 h dropped to 0.25 ± 0.07 μg/ml, and only traces were detected in samples taken 24 h after dosing.

The uterine weight of 17β-E_2-treated rabbits was 19-fold higher than that of the control animals (**Table 2**). The uterine concentration of ERα after estradiol treatment was significantly lower. The apparent cytosolic:nuclear distribution of ERα, however, was not significantly different compared with the controls.

In the naringenin group, the uterine weight, the total concentration of Erα, and the apparent cytosolic:nuclear distribution of ERα were not significantly different than those in the control group.

The 17β-E_2-treated rabbits displayed significantly lower relative liver weight and GST activity, whereas the UDPGT activity was not affected compared with the control group (**Table 3**).

In the naringenin group, the relative liver weight, and the activities of GST and UDPGT, were not significantly different from those in the control group.

TABLE 2. Uterine weight, and concentration and percent-wise distribution of ERα in cytosol and nuclei from uterus, of ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

Group/Diet	Uterus	ERα			ERα	
		Cytosol	Nuclei	Total	Cytosolic Fraction	Nuclear Fraction
	g/kg body weight	*fmol/mg cytosolic protein*			%	
I. Control	0.22 ± 0.13	34 ± 10	7.8 ± 7.7	42 ± 11	83 ± 16	17 ± 16
II. 0.004% 17β-E_2	4.1 ± 3.1[a]	10 ± 6[a]	1.7 ± 1.5[b]	12 ± 7[a]	89 ± 9	11 ± 9
III. 0.2% naringenin	0.18 ± 0.26	44 ± 23	6.6 ± 7.2	50 ± 30	90 ± 8	10 ± 8

[a] $P < 0.001$, compared with the control group, Student's *t*-test on logarithmically transformed data.
[b] $P < 0.01$, compared with the control group, Student's *t*-test on logarithmically transformed data.

TABLE 3. Relative liver weight and activity of phase 2 enzymes in liver tissue from ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

Group/Diet	Relative Liver Weight	GST	UDPGT
	g/kg body weight	*μmol glutathione conjugate/ min/g protein*	*μmol umbelliferyl/ min/mg protein*
I. Control	23.4 ± 9.0	1,800 ± 300	0.376 ± 0.198
II. 0.004% 17β-E_2	17.4 ± 3.4[a]	1,300 ± 400[a]	0.343 ± 0.149
III. 0.2% naringenin	23.1 ± 8.0	1,700 ± 600	0.239 ± 0.086

[a] $P > 0.05$, compared with the control group, Student's *t*-test.

Effect of test compounds on plasma lipids, oxidation level, and antioxidant enzyme activity in erythrocytes

In the 17β-E_2 group only a transitory decrease in plasma cholesterol (week 12, $P < 0.05$) and a significant increase in HDL cholesterol were recorded when compared with the controls (**Table 4**). Plasma triglyceride concentrations were not significantly different from those in the controls, but the lower total triglyceride concentration reached a borderline significance ($P = 0.0534$) by week 16. Also, significantly lower levels of LDL and IDL triglycerides were observed by week 16 compared with the control group.

In the naringenin group a significantly increased HDL cholesterol level was the only observed variation in the lipid profile when compared with the controls.

The oxidation level expressed as a concentration of MDA in the 17β-E_2 group was significantly increased in LDL, but not in plasma, when compared with the controls (**Table 5**). The activities of the antioxidant enzymes in erythrocytes were comparable to those in the control group.

In the naringenin group, the concentrations of MDA in plasma and LDL, and the activities of SOD, catalase and GPx. were not significantly different compared with the control group. The GR was significantly downregulated.

Effect of test compounds on aortic atherosclerosis

The aortic cholesterol content in the 17β-E_2 group was significantly lower in the ascending aorta and in the total aorta compared with the control group (**Table 6**). In contrast, neither the ratio intima:media nor the area of intima was significantly different from those in the control group (**Table 7**). The number of animals with no lesion was significantly increased in the abdominal aorta only.

In the naringenin group, all aortic atherosclerosis parameters were not significantly different from those in the controls (Tables 6 and 7).

A weakly positive, but significant, correlation between aortic cholesterol accumulation and time-averaged plasma cholesterol was detected in the control group but not in the two other groups (**Fig. 2**).

DISCUSSION

In contrast to several studies that demonstrated that 17β-E_2 decreases development of experimental atherosclerosis with or without lowering of hypercholesterolemia

TABLE 4. Blood lipids in ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

	No. of Animals	Week of Treatment					Concentration in Lipoproteins in Week 16			
		0	4	8	12	16	HDL	LDL	IDL	VLDL
		mM					*mM*			
Cholesterol										
I. Control										
Mean	12	25.4	33.2	33.5	40.5	35.8	0.53	12.74	8.03	10.50
SD		3.8	7.1	7.5	6.1	6.5	0.31	2.69	3.53	4.95
II. 0.004% 17β-E_2										
Mean	13	26.9	34.1	31.8	35.1[a]	34.7	1.08[b]	11.20	6.97	10.90
SD		3.7	3.6	4.6	4.8	6.2	0.57	2.09	2.24	4.33
III. 0.2% naringenin										
Mean	11	24.4	34.5	36.8	39.7	39.2	1.02[c]	11.86	9.09	13.35
SD		2.7	3.8	5.8	6.0	6.6	0.18	3.08	2.77	4.84
Triglycerides										
I. Control										
Mean	12	6.30	1.90	1.56	1.74	2.35	0.14	0.71	0.48	0.78
SD		1.87	0.74	0.58	0.88	1.54	0.04	0.43	0.35	0.68
II. 0.004% 17β-E_2										
Mean	13	7.12	1.54	1.19	1.33	1.39[d]	0.15	0.35[a]	0.23[a]	0.49
SD		2.38	0.52	0.40	0.53	0.70	0.08	0.16	0.17	0.30
III. 0.2% naringenin										
Mean	11	5.58	1.60	2.28	1.96	2.20	0.16	0.55	0.42	0.79
SD		2.55	0.52	1.43	1.20	1.66	0.05	0.44	0.38	0.68

[a] $P < 0.05$, compared with the control group, repeated measure analysis on plasma cholesterol data, and Student's *t*-test on LDL and IDL triglyceride logarithmically transformed data.
[b] $P < 0.01$, compared with the control group, Student's *t*-test on logarithmically transformed data.
[c] $P < 0.001$, compared with the control group, Student's *t*-test on logarithmically transformed data.
[d] $P = 0.0534$, compared with the control group, repeated measure analysis.

TABLE 5. Oxidation level in plasma and LDL determined as total MDA and activity of the antioxidant enzymes in erythrocytes of ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

	MDA		Red Blood Cell Antioxidant Enzymes			
Group/Diet	Plasma	LDL	SOD	GR	Catalase	GPx
	nmol/g protein		*U/g hemoglobin*			
I. Control	92 ± 14	381 ± 87	1,700 ± 300	8.64 ± 2.04	8.90 ± 1.41	182 ± 116
II. 0.004% 17β-E_2	97 ± 14	489 ± 147[a]	1,900 ± 400	8.33 ± 1.23	9.20 ± 1.70	176 ± 95
III. 0.2% naringenin	92 ± 19	413 ± 84	1,500 ± 300	6.94 ± 1.69[a]	9.01 ± 1.75	155 ± 121

[a] $P < 0.05$, compared with the control group, Student's *t*-test (GR data not transformed, MDA in LDL data logarithmically transformed).

in cholesterol-fed rabbits (1–3, 28, 29), no information is available about the effect of this estrogen on blood lipids and development of spontaneous atherosclerosis in WHHL rabbits. Both models differ in more than the origin of hypercholesterolemia when considering their application in studies of the antiatherogenic effect of estrogens and phytoestrogens. The major difference is that the atheroprotective action of estrogens, such as enhanced clearance of atherogenic lipoproteins due to an upregulation of liver LDL receptors, is excluded in WHHL rabbits because of the deficiency of this receptor. In addition, whereas sexually mature ovariectomized rabbits were used for studies of the cholesterol-fed model, young, sexually immature ovariectomized WHHL rabbits were used in this study. This is due to the specific characteristics of this model. Spontaneous atherosclerosis in WHHL rabbits develops and progresses with age. Assuming that lesions cannot be prevented once started, but may be retarded, it was decided to use animals no more than 8 weeks old, as their aortas are free from lesions (by light microscopy) at this age (16).

The recorded increased body weight at termination in all groups indicates no adverse effect of the test compounds on this parameter. The slightly (but not significantly) decreased body weight, together with a significantly decreased relative feed intake in the 17β-E_2 group at termination compared with the control group, could either be due to the lower palatability of this diet or to the reduction of both clinical parameters by estrogens as previously suggested (30).

The significantly increased uterine weight in the 17β-E_2 group verified the in vivo 17β-E_2 action. The plasma levels of estradiol in the control and naringenin groups were comparable to those previously reported in ovariectomized untreated cholesterol-fed rabbits (2, 3). The atrophic uteri with a low weight in the control group confirmed the absence of endogenous estrogenic stimulation and verified complete ovariectomy. In the naringenin group the atrophic uteri with weights similar to those in the controls indicated no stimulatory effect of the test compound on endometrium, in accordance with no effect of naringenin on the uterine weight in immature rats (31). The findings of decreased uterine concentration of

TABLE 6. Quantitative biochemical evaluation of aortic atherosclerosis in ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

Cholesterol Content	I. Control	II. 0.004% 17β-E_2	III. 0.2% Naringenin
nmol/cm²			
Ascending aorta	4.70 ± 1.50	3.48 ± 1.21[a]	3.95 ± 1.01
Thoracic aorta	1.88 ± 1.05	1.42 ± 1.02	2.38 ± 1.55
Abdominal aorta[b]	1.67 ± 0.61	1.19 ± 0.68	1.95 ± 1.52
Total aorta	8.26 ± 2.65	5.88 ± 2.50[a]	8.28 ± 3.80

[a] $P < 0.05$, compared with the control group, Student's *t*-test.
[b] One biochemical sample from abdominal aorta in the 17β-E_2 group was lost due to a technical error.

TABLE 7. Incidence of aortic atherosclerotic lesions and quantitative microscopic evaluation of aortic atherosclerosis in ovariectomized WHHL rabbits untreated or given 17β-E_2 or naringenin in the diet for 16 weeks

Group/Diet	I. Control	II. 0.004% 17β-E_2	III. 0.2% Naringenin
Ascending aorta			
No. of animals with no lesion	1/12	1/13	0/11
Intima:media			
Mean ± SD	0.30 ± 0.18	0.25 ± 0.22	0.36 ± 0.17
Median	0.34	0.19	0.35
Range	0–0.58	0–0.78	0.08–0.65
Area of intima (mm^2)			
Mean ± SD	1.63 ± 0.94	1.31 ± 1.35	2.01 ± 1.16
Median	1.78	0.85	1.78
Range	0–2.96	0–4.8	0.33–4.01
Thoracic aorta			
No. of animals with no lesion	3/12	7/13	0/11
Intima:media			
Mean ± SD	0.18 ± 0.15	0.15 ± 0.25	0.44 ± 0.40
Median	0.19	0	0.41
Range	0–0.45	0–0.66	0–1.3
Area of intima (mm^2)			
Mean ± SD	0.38 ± 0.34	0.39 ± 0.66	0.99 ± 1.11
Median	0.36	0	0.69
Range	0–1.05	0–2.1	0–3.75
Abdominal aorta[a]			
No. of animals with no lesion	0/12	5/13[b]	0/10
Intima:media			
Mean ± SD	0.30 ± 0.20	0.21 ± 0.25	0.39 ± 0.25
Median	0.28	0.05	0.33
Range	0.05–0.68	0–0.67	0.07–0.9
Area of intima (mm^2)			
Mean ± SD	0.45 ± 0.29	0.34 ± 0.45	0.58 ± 0.36
Median	0.46	0.07	0.53
Range	0.07–0.99	0–1.18	0.13–1.25

[a] One histological sample from the abdominal aorta in the naringenin group was lost due to technical error.
[b] $P < 0.05$, compared with the control group, Fisher's exact test.

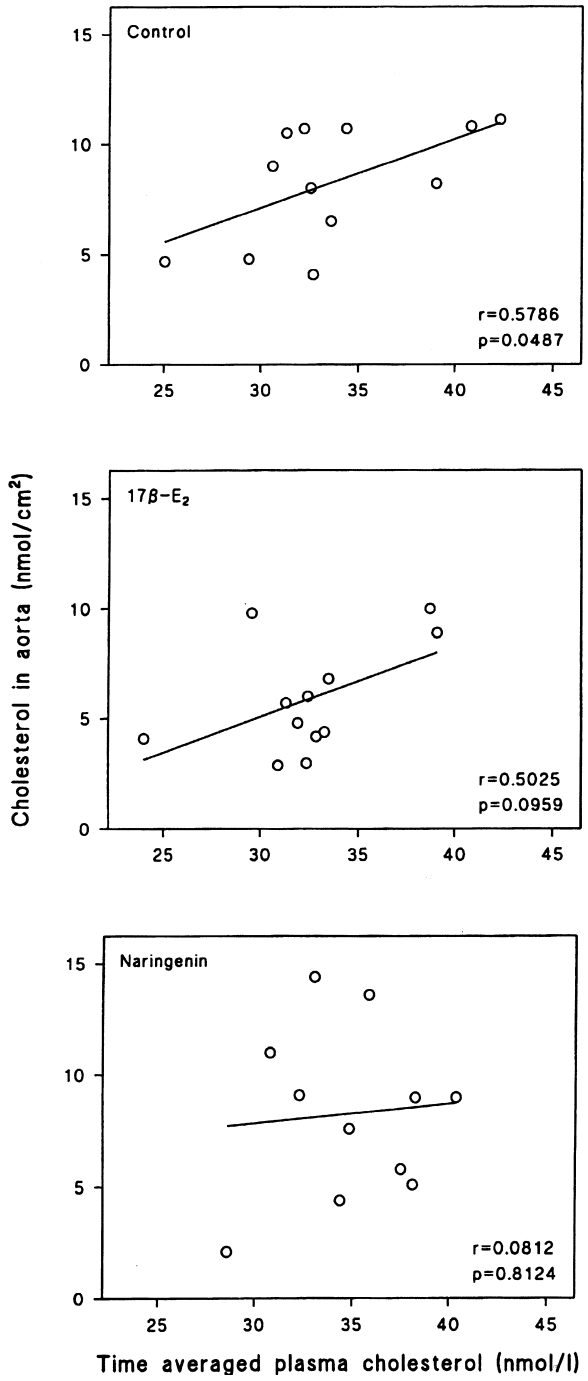

Fig. 2. A weak but significant correlation between total aortic cholesterol accumulation and overall plasma cholesterol exposure, expressed as time-averaged plasma total cholesterol, was found in the control group but not in the 17β-E_2 and naringenin groups.

ERα in the 17β-E_2-treated rabbits are in accordance with the previously reported observation of an increase after administration of moderate doses of estrogen, and a decrease after administration of high doses (32).

The determination of the activity of GST and UDPGT in the liver was included in this study because general interest in possible health beneficial effects of flavonoids is not limited to their atheroprotection. It also concerns their potential anticarcinogenic properties connected to the ability to modulate phase 1 and phase 2 enzymes in preparations from experimental animals or humans. The significant decrease in hepatic GST activity and in relative liver weight was observed in the present study after estradiol administration. The mechanism responsible for this is unknown. However, the decrease in hepatic GST of approximately 30% should be regarded with major concern. As the GST isozymes constitute one of the major groups of enzymes involved in the detoxification of both endogenous and exogenous chemicals, a decreased activity of GST in the liver might result in impaired resistance to toxic agents such as oxidants or chemical carcinogens. Information about whether hepatic or red blood cell GST is also downregulated in humans in response to estrogen treatment (thereby rendering the individual more susceptible to encountered electrophilic compounds) is greatly needed. The observation of no induction of GST in the liver of naringenin-treated rabbits is in accordance with previously published observations of hydroxylated or metoxylated flavonoids (33) and with the observation of no effect of this flavonoid on GST and UDPGT in the rat liver (34).

Even though the flavonoids are generally regarded as good antioxidants [an assumption based on in vitro studies (35, 36)], little is known about their actual in vivo capacity to function as antioxidants. The same relates to the potential antioxidant effects of 17β-E_2, as most of its antioxidant action has been demonstrated in vitro as an antioxidant protection on LDL (37–39). The significant increase in total amount of MDA in LDL in the 17β-E_2 group, compared with the controls, indicates that 17β-E_2 enhanced the oxidative modification of LDL. This has been previously suggested and proposed as a part of the antiatherogenic action of 17β-E_2 (37) in accordance with "removal of LDL by oxidation" (19).

The absence of significant differences in concentrations of MDA in plasma and LDL and in the activity of red blood cell antioxidant enzymes, except for GR, between the naringenin and control groups does not support an in vivo antioxidant potential for naringenin. This is in accordance with previous findings in rats (34).

It is well established that estrogen replacement therapy is associated with beneficial alteration in several lipoproteins, including a reduction in LDL cholesterol and an approximately 10%–15% increase in HDL cholesterol (37, 40). However, information about the effect of phytoestrogens on plasma lipoproteins appears limited to the lowering of cholesterol in animals by soy protein, which is partially ascribed to soy isoflavones (41). The beneficial alteration of the cholesterol concentration in lipoproteins by 17β-E_2 and naringenin in this study was limited to the significantly increased HDL cholesterol, which was higher then expected. No decrease in atherogenic lipoproteins should be regarded as a consequence of LDL receptor deficiency in this model (reduced uptake and degradation by the liver).

The high initial plasma triglyceride concentration in all the groups is in accordance with the previously described blood lipid levels in young homozygous WHHL rabbits from this colony (42, 43), as well as with those recorded at

the start of other intervention studies (44, 45). The observed decrease in plasma triglycerides, beginning 4 weeks from the start and throughout the study in all groups, can be explained by the difference in fat ingested before and after the start of the study. As suckling pups, all the rabbits received approximately 13% fat in their mother's milk. From 6 weeks of life until the start of the treatment, they ingested approximately 3.5 g of fat daily (average mean feed intake per rabbit of 100 g of breeding rabbit diet Altromin 2113) whereas their daily fat intake during the study was 1.5–1.8 g/kg body weight depending on the treatment group, as determined on the basis of the recorded feed intake. The increase in plasma triglyceride concentration due to increased hepatic triglyceride secretion in the form of increased entry of VLDL into the circulation, seen after oral estrogen administration in humans, was not recorded in this study. In fact, the plasma triglyceride level at the end of the study in the 17β-E_2 group was slightly decreased ($P = 0.0534$). This slight decrease was accompanied by recorded significantly decreased triglycerides in LDL and IDL. It should be added that no increase in plasma triglycerides after 17β-E_2 treatment has been recorded in the cholesterol-fed rabbit model (2, 3, 46).

The protective effect of 17β-E_2 on development of experimental aortic atherosclerosis in rabbits (1–3, 29, 47, 48) and in mice (5, 6) fed various amounts of cholesterol in the diet is well documented. However, two of the studies of cholesterol-fed rabbits present data on significantly decreased aortic atherosclerosis in the aortic arch only (inner proximal layer portion up to the level of the first intercostal arteries of the thoracic aorta) and no information is given about aortic cholesterol accumulation in the thoracic aorta distal from the first intercostal arteries and in the abdominal aorta (2, 3). The decrease in atherosclerosis recorded by quantification of lipid-positive area was limited to the thoracic aorta including the arch (29). In a study of the effect of 17β-E_2 on pre-existing atherosclerotic lesions in cholesterol-fed rabbits, the severity of atherosclerosis was measured as intima in square millimeters, and it was demonstrated to be significantly lower in the thoracic aorta but not in the aortic arch and abdominal aorta (48). In another study of cholesterol-fed rabbits, the decrease in aortic atherosclerosis in 17β-E_2-treated rabbits compared with the controls was demonstrated by visual evaluation (1). However, it was not clear whether the evaluation included the whole aorta or chosen parts only. The quantitative biochemical, microscopic, and macroscopic methods for evaluation of aortic atherosclerosis used in the mentioned studies, and in the present study, were previously evaluated in this laboratory (16). It is important to note that measurement of the aortic cholesterol content is a combined measurement of atherosclerosis extent and severity. The quantitative microscopic evaluation is largely a measure of the severity of aortic atherosclerosis, whereas macroscopic visual evaluation (the most imprecise method) and quantitation of the aortic lipid-positive area are both a measure of the extent of the disease. In the present study, administration of 17β-E_2 (but not phytoestrogen naringenin) significantly reduced cholesterol accumulation in the ascending aorta and in the whole aorta but not the severity of atherosclerosis evaluated microscopically. The findings on aortic cholesterol accumulation in the ascending aorta of WHHL rabbits in this study are comparable to those in cholesterol-fed rabbits. Furthermore, the significantly decreased aortic cholesterol accumulation in the ascending aorta and in the total aorta in the 17β-E_2 group indicates an atheroprotective effect of the compound. No difference in the total plasma cholesterol between the control and 17β-E_2 groups, and no correlation between the cholesterol accumulation in the entire aorta and overall cholesterol exposure expressed as time-averaged plasma total cholesterol, in 17β-E_2 group (Fig. 2), demonstrate that the protective action of 17β-E_2 in WHHL rabbits was independent of cholesterol lowering, in accordance with findings in LDL receptor-deficient mice (6).

Furthermore, the protective action was not due to lowering of LDL cholesterol; it could be due to the earlier suggested limitation of retention of lipoprotein in the arterial wall (41). On the basis of the present results, however, it could also be linked to the recorded increased oxidation in LDL and the increased HDL cholesterol. Although 17β-E_2 does not reduce the permeability of the aortic wall to LDL (49, 50), it decreases the accumulation of oxidized LDL (51) in it. This could lead to a reduced uptake of oxidized LDL by macrophage foam cells and therefore attenuate the cholesterol accumulation in the aortic wall. The ability to increase HDL cholesterol is a recognized mechanism by which estrogens are thought to protect against atherosclerosis. HDL accepts tissue-derived free cholesterol, the availability of which has been suggested to be increased by 17β-E_2 and other estrogens (37), and delivers it as a cholesteryl ester to the liver directly or after transfer to apolipoprotein B-100 (apoB-100)-containing lipoproteins. In the system with impaired uptake of apoB-100-containing lipoproteins (LDL and IDL) the direct transport of cholesteryl ester by HDL to the liver could play an important role in the reverse cholesterol transport.

The lack of an effect of naringenin on aortic atherosclerosis at the dose level significantly exceeding the estimated daily human intake of 2.4 g (13) indicates the absence of an atheroprotective action of this phytoestrogen. However, the relevance of this finding for humans, especially for postmenopausal women, should be regarded with caution. Keeping in mind that the estrogen receptor binding affinity for naringenin is 10 times greater for ERβ than for ERα (52) and given that humans have about equal abundance of ERβ and ERα in arterial tissues, the information about ERβ abundance in aorta of WHHL rabbits would be helpful for extrapolation to human situation. Furthermore, naringenin exhibits antiestrogenin activity in vivo (31). In rats it was shown to inhibit the 17β-E_2-induced increase in uterine weight, induction of progesterone receptor binding, [^{3}H]thymidine uptake, and uterine peroxidase activity. Naringenin also attenuated the estrogen-induced increase in cell proliferation in MCF-7 human breast cancer cells (31). The antiestrogenic activity of naringenin was suggested as one mechanism as-

sociated with the activities of dietary flavonoids as inhibitors of mammary cancer in rodent studies and the decreased incidence of breast cancer in woman from countries with high consumption of phytoestrogens. The effects of the antiestrogenic activity of naringenin (and other dietary phytoestrogens) on atheroprotective effects of estrogens remains to be established. This might be of importance for future dietary guidance, especially of postmenopausal woman receiving hormone replacement therapy.

17β-E_2 can exert an atheroprotective effect by several mechanisms [reviewed in refs. (37), (40), and (41)]. An earlier study of LDL receptor knockout mice fed cholesterol (6) demonstrated that 17β-E_2 can reduce the extent of aortic lipid deposition. The present study of WHHL rabbits demonstrated that 17β-E_2 can attenuate cholesterol accumulation without affecting the total and LDL plasma cholesterol. Both studies demonstrated that LDL receptor deficiency did not abolish the atheroprotective action of 17β-E_2. Furthermore, the results from the present study support the previously suggested pro-oxidant ability of 17β-E_2 toward LDL (37) and indicate the possible connection between the pro-oxidant nature of 17β-E_2 and its antiatherogenic effect. However, this requires further investigation. The challenge for the future is to obtain a better understanding of the cellular and molecular mechanisms by which 17β-E_2 and other estrogens exert their atheroprotective effect and to identify natural phytoestrogens with antiatherogenic potential. The WHHL rabbit seems to be an appropriate model to be used along with other animal models in this field of research.

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