Tibolone Prevents Atherosclerotic Lesion Formation in Cholesterol-Fed, Ovariectomized Rabbits

Pieter Zandberg, Jan L.M. Peters, Pierre N.M. Demacker, Martin J. Smit, Ernst G. de Reeder, Dirk G. Meuleman

Abstract—Tibolone (Org OD14), a synthetic steroid with estrogenic and progestogenic/androgenic properties, is clinically effective for the treatment of climacteric symptoms and the prevention and treatment of osteoporosis in postmenopausal women. The effect on atherogenesis, however, is not known. In the current study, we investigated the effect of tibolone in comparison with that of estradiol and norethisterone acetate on atherogenesis in 140 ovariectomized New Zealand White rabbits that had been induced by an atherogenic diet (0.4% cholesterol, 20 weeks). Tibolone at 18, 6, or 2 mg/d orally completely prevented cholesterol accumulation and fatty streak formation in the aorta; the impairment of endothelium-dependent smooth muscle relaxation of the aorta; and complex lesion formation after endothelial denudation in the carotid artery. Tibolone also reduced the increased postovariectomy plasma lipid concentrations. Analysis of the results, however, indicated that a substantial part of the strong, beneficial effects were plasma lipid independent. Compared with subcutaneous estradiol decanoate (150 μg once weekly) and oral 17β-estradiol (4 mg/d), the effects of tibolone were more pronounced at equipotent uterotrophic activity. Norethisterone acetate (1 mg/d) did not affect atherosclerotic lesion formation. There are no indications that the progestogenic/androgenic properties of tibolone counteracted its atheroprotective effect on the vessel wall. Therefore, tibolone has the intrinsic potential to be a compound that protects the arterial vessel wall against atherosclerotic processes. (Arterioscler Thromb Vasc Biol. 1998;18:1844-1854.)

Key Words: lipids ■ aorta ■ sex hormones ■ Org OD14 ■ atherogenesis

There is considerable epidemiological evidence that estrogen deficiency as it occurs at menopause increases women’s risk of cardiovascular disease, in particular coronary heart disease (CHD), and that estrogen replacement can prevent this.1–4 Originally, estrogen’s effects were primarily attributed to its plasma cholesterol– and LDL cholesterol–lowering and/or plasma HDL cholesterol–increasing effects.5,6 Currently, there is strong evidence that as much as 50% to 75% of the beneficial effects of estrogen replacement therapy (ERT) on CHD is of nonplasma lipid origin.1,6–11 In particular, animal studies8,12–19 have provided evidence that lipid-independent mechanisms are playing a role in atheroprotection. These include direct effects on the vessel wall,8,16,20,21 suppression of cholesterol deposition,13 inhibition of LDL oxidation,22 inhibition of proliferation of intimal cells13,23 and the synthesis of extracellular matrix,24 and preservation of vascular responsiveness.25–28 Some effects on vessel wall functionality are presumably nongenomic, whereas the other effects of estrogen are probably due to alterations in gene expression.11

Clinically unopposed ERT in postmenopausal women can lead to endometrial hyperplasia. Therefore, a progestagen is frequently added to the estrogen therapy to negate these estrogen-mediated risks. The consequences of such combined hormone replacement therapy on the occurrence of CHD are controversial.3,4,6,29–32 Progestagens can have adverse effects on lipoprotein concentrations33,34 and negatively affect the beneficial effects of estradiol on atherosclerotic lesion formation.35–38 Other investigations, however, showed no negative effect of progestagens on the beneficial effects of estrogen on the vessel wall.13,14,19

Tibolone (Org OD14), a synthetic steroid [(7α,17α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one] with a combination of estrogenic, androgenic, and progestogenic properties, is clinically effective for the treatment of climacteric symptoms39,40 and the treatment and prevention of osteoporosis in postmenopausal women,41 with no stimulatory effect on the endometrium.42,43 The effect on the development of atherosclerosis is not known. It has been suggested that tibolone might have less atheroprotective effect than ERT because of its progestogenic/androgenic properties.44

We therefore investigated the effect of tibolone on atheromatous lesion formation in ovariectomized (OVX) rabbits that were fed a diet enriched with cholesterol in comparison

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with unopposed subcutaneously injected depot preparation estradiol decanoate (E2-D), oral 17β-estradiol (E2), the progestagen norethisterone acetate (NETA), and a combination treatment of E2/NETA. Other investigators 12–15 have shown that this animal model allows assessment of the lipid-independent effect of hormone replacement therapy on atherogenesis.

Methods

Animals and Experimental Design

The current study was conducted in accordance with the guidelines for animal research of the Dutch government and were approved by the Animal Care and Use Committee of NV Organon. One hundred forty sexually mature, virgin female New Zealand White rabbits (Harlan, Zeist, The Netherlands), aged 7 to 9 months and weighing ~3 kg, were used. During the acclimatization period, the rabbits were fed a diet of standard commercial rabbit chow LKK20 (Hope Farms). Three weeks before the start of the experiment, 126 animals underwent bilateral ovariotomy and 14 animals were sham-operated. For this procedure, the animals were anesthetized with 5 mg/kg xylazine (Sedamun, AUG) and 35 mg/kg ketamine (Aescoket, Aesculaap BV), injected intramuscularly. The local anesthetic lidocaine (AUG, Cuyk) was injected locally (intramuscularly and subcutaneously) in the surgical area. To prevent wound infections, 1 mL of the antibiotic Albipen (Mycopharm) was administered subcutaneously. After 3 weeks, at the start of the experiment, the rabbits were randomized into 10 groups (Table 1). In all animals, deendothelialization of a segment of the left carotid artery was performed by using the air-drying technique.45,46 A 2-cm segment of the left carotid artery of the anesthetized animals (see above) between 2 ligatures was emptied of blood by applying two 27-gauge needle punctures. The segment was flushed with physiological saline, and then an airflow of 250 mL/min for 5 minutes was applied. Puncture holes were covered with Willospon (Will-Pharma), and blood flow was restored. The air-dried portion was indicated with 2 hemoclips.

The animals were randomly allocated to 10 experimental groups (see Table 1) by using a randomized block design of 7 blocks. Nine groups were fed an atherogenic diet (commercial rabbit chow LKK20 enriched with 0.4 g cholesterol, 3.75 g coconut oil, and 3.75 g peanut oil per 100 g of chow), and 1 group was continued on the standard rabbit chow (LKK20). Food intake was restricted to 80 g daily. Except for group 4 (E2-D), treatments were administered orally as a tablet containing basic granulate lactose (100 mg) and dried potato starch (10 mg). Group 4 (the reference group) was given 1.10 g/mL fraction

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Treatment</th>
<th>Dose</th>
<th>Diet</th>
<th>O VX</th>
<th>Number of Animals at Autopsy</th>
</tr>
</thead>
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<tr>
<td>1 Pl.o</td>
<td>14</td>
<td>Placebo</td>
<td></td>
<td>Cholesterol</td>
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<td>9 (11)*</td>
</tr>
<tr>
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<td>13</td>
<td>Placebo</td>
<td></td>
<td>Cholesterol</td>
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<td>11</td>
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<tr>
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<td>13</td>
<td>Placebo</td>
<td></td>
<td>Normal</td>
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<td>13</td>
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<td>14</td>
<td>17β-Estradiol decanoate</td>
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<tr>
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<td>4 mg</td>
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<td>13</td>
</tr>
<tr>
<td>6 E2/NETA</td>
<td>13</td>
<td>E2/NETA</td>
<td>4 + 1 mg</td>
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<td>13</td>
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<tr>
<td>7 NETA</td>
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<tr>
<td>8 T.l</td>
<td>13</td>
<td>Tibolone</td>
<td>2 mg</td>
<td>Cholesterol</td>
<td>Yes</td>
<td>13</td>
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<tr>
<td>9 T.m</td>
<td>14</td>
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<td>6 mg</td>
<td>Cholesterol</td>
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<td>10 T.h</td>
<td>14</td>
<td>Tibolone</td>
<td>18 mg</td>
<td>Cholesterol</td>
<td>Yes</td>
<td>14</td>
</tr>
</tbody>
</table>

Pl indicates placebo; o, OVX; n, non-OVX; Ctrl, control; T, tibolone; l, low; m, medium; and h, high.

The treatments were administered for a period of 20 weeks orally once a day except for group 4 (17β-estradiol decanoate [E2-D]) in which the dose was injected subcutaneously once a week. After 20 weeks, autopsy was performed and the different organs and/or arteries were removed for further experiments and/or analyses. During the experiment, there were several dropouts. The number of animals that completed the study are presented in the last column.

*Two animals in a moribund state that were killed during week 19 were included in the autopsy but not in the biochemical and functional measurements.

Blood Biochemistry

Blood samples were drawn from the central ear artery after sedation with Hypnorm (0.1 mL IM; Janssen Pharmaceutica) before the daily treatment to monitor plasma lipid and lipoprotein concentrations, concentrations of glutamate pyruvate transerase (GPT), bilirubin, and blood cell counts during the experiment. Bilirubin, GPT, triglycerides, and total plasma cholesterol measurements were performed at 30°C on an Encore centrifugal analyzer using Boehringer test kits (Boehringer Mannheim).

For the assessment of plasma lipoprotein cholesterol distribution, blood was collected in 0.03 volume 5% EDTA. To the plasma, sucrose was added as a cryoprotective agent (final concentration, 5% wt/vol; J.T. Baker BV, catalog No. 0334). Samples were stored at −80°C until use. The β-VLDL plus IDL fractions were isolated as the d<sub>1.019</sub> g/mL fraction after ultracentrifugation by using a Sorvall TST 45.6 rotor in a Beckman L7-55 ultracentrifuge for 16 hours at 120 000g at 15°C.47 HDL fractions were isolated by precipitating the LDL from the d>1.019 g/mL (LDL+HDL) fraction with PEG 6000.48 HDL<sub>d</sub> was isolated as the d>1.10 g/mL fraction obtained by ultracentrifugation of plasma for 24 hours at 138 000g in the TFT 45.6 rotor at 15°C. Total plasma and lipoprotein-associated cholesterol was determined on a Hitachi 747 analyzer using the enzymatic CHOD-PAP method (Boehringer Mannheim, catalog No. 237574) and Preciset cholesterol (catalog No. 125512, Boehringer Mannheim), for calibration. LDL cholesterol and HDL<sub>d</sub> cholesterol were calculated by subtraction.

Hormone Levels

Tibolone is rapidly metabolized into the 3α- and 3β-hydroxy metabolites with estrogenic properties and the Δ4 isomer with
progestogenic/androgenic properties. The plasma concentrations of 17β-estradiol and of tibolone and its metabolites were determined in samples obtained during week 17 at 1, 2, 4, 8, and 24 hours after administration as follows.

For the determination of 17β-estradiol, plasma was extracted (solid-phase extraction), recollected in methanol, and stored at −20°C until use. After evaporation in a VAC Elut SPS-24 (Speed Vac, Béton Scientific), the residue was resuspended in estradiol “0” standard buffer (ICN Biomedicals Inc), and estradiol content was determined by using a radioimmunoassay kit (ImmuChem double-antibody 17β-estradiol 125I RIA kit; Campro Scientific ICN).

For determination of tibolone, an internal standard (2H₃ form of the D isomer, the internal standard (2H₅ form of the D₄ isomer) was added to the plasma samples immediately after sampling to correct for tibolone instability. For determination of the tibolone) was added to the plasma samples. The samples were processed with C₁₈ solid-phase extraction, and after Tri-Sil derivatization and reconstitution in water, they were reextracted with n-hexane. The n-hexane phase was transferred and evaporated to dryness. The residue was redissolved in ethanol, evaporated to dryness, and redissolved in isooctane, from which an aliquot was analyzed by capillary gas chromatography–mass spectrometry. For determination of the 3α- and 3β-hydroxy metabolites of tibolone, an internal standard (2H₃ form of the 3α-hydroxy metabolite) was added to the plasma samples. The samples were processed with C₁₈ solid-phase extraction, and after Tri-Sil derivatization and reconstitution in water, they were reextracted with n-hexane. The n-hexane phase was transferred and evaporated to dryness. The residue was redissolved in ethanol, from which an aliquot was analyzed by capillary gas chromatography–mass spectrometry.

Calibration curves were constructed using a weighted linear regression. From the calibration curves, the concentrations in the study samples were calculated.

Necropsy
Twenty weeks after the start of the experiment, the animals were anesthetized by an injection of Hypnorm (0.5 mL/kg IM). After blood sampling, the rabbits were killed by exsanguination, and the various organs and/or arteries were removed for further experiments or analyses. The weights of the liver and uterus were determined.

Vascular Reactivity
At necropsy, the descending thoracic aorta was dissected free and placed in an oxygenated physiological salt solution (see below). Collagen and fat were removed. Care was taken not to touch the luminal endothelium. A ring with a length of 3 to 5 mm was suspended between 2 stainless steel hooks inserted into the lumen of the ring for the measurement of isometric tension in individual organ baths containing 5 mL of a physiological salt solution at 37°C aerated with 95% O₂ and 5% CO₂. The composition of the physiological salt solution was as follows (in mmol/L): NaCl 118, KCl 5.9, CaCl₂ 2.2, MgSO₄·7H₂O 1.2, NaH₂PO₄·H₂O 1.2, NaHCO₃, 25.0, and D-glucose 5.6. The pH after aeration was 7.4. The resting tension concentration of animal on treatment i; xᵢ= the individual mean log of vessel wall cholesterol level for the control animals; and xᵢ= the mean total plasma cholesterol level for the control animals. The calculated F values in the ANOVA were used for the P values of significance.

Results
General
Fourteen animals per group entered the study. During the stabilization and randomization periods, however, some animals were excluded because of severe weight loss (1), hindpaw infection (2), or complications during the air-drying procedure (2). Therefore, 135 animals divided into 10 groups entered the actual study period (see Table 1). Except for the control group (group 3), the rabbit chow was replaced by the atherogenic diet. After some accommodation in the first week, the animals completely consumed the offered food.

Evaluation of Aortic Atherosclerosis
For measurement of fatty streaks, the aortas were dissected free and divided into 3 parts: aortic arch, thoracic aorta, and abdominal aorta. The aortic tissue was opened longitudinally, fixed in 2% paraformaldehyde, and stained for lipids with 0.3% (wt/vol) Sudan red. Color photographs were taken of all segments, and the percentage of coverage with fatty streaks was assessed by using image analysis (Context Vision Systems AB). After fatty streak measurement, the 3 parts were minced in a dismembrator (Mikro-Dismembrator, B. Braun) followed by lipid extraction according to the method of Bligh and Dyer. Total cholesterol content was determined with the enzymatic CHOD-PAP method (catalog No. 1442341, Boehringer Mannheim). The amount of protein in the tissue was determined by the method of Lowry et al.

The left (air-dried) carotid artery was dissected and fixed in 2% paraformaldehyde containing 6.8% glucose. The right carotid artery was used for comparison. After fixation, the tissue was divided in 2-mm-long blocks and embedded in paraffin (Paraplast plus, Sherwood Medical Co). Measurement of intimal thickness was performed on 2-μm transverse sections that were treated with elastase (Serva Feinbiochemica GmbH) before elastin staining with Lawson solution (Boom) and light green SF yellowish (Sigma). Subsequently, sections were air-dried and mounted in Pertex (Leica GmbH). For morphological study, both methylene blue/azure II– and hematoxylin/eosin–stained (2-μm) transverse sections were used. Smooth muscle cells and macrophages were detected with α-actin antibodies (Sigma) and anti-macrophage antibodies (RAM11, DAKO, respectively. For detection of bound antibodies, goat anti-mouse ultra-small gold-conjugated secondary antibodies (Au- tion) and the immunogold-silver enhancement technique (SilvEnhance-LM kit, Zymed) were used. Images of the sections were obtained with a black-and-white video camera (MX-5, Adimec Image Systems BV) mounted on a light microscope (Axioplan, Zeiss). The video image was digitized, and the intimal thickening was measured by using a semiautomated image analysis application software (Context Vision Systems AB).

Statistics
Data are expressed as mean±SE unless otherwise specified. For testing statistical significance, ANOVA was used. The data were logarithmically transformed to normalize variations. If ANOVA indicated significant differences between groups, Student’s t test was used to test the treatments pairwise. A value of P<0.05 was considered significant.

To test whether vessel wall cholesterol concentration was a function of plasma lipids, we applied regression analysis. Linear regression analysis showed a high correlation between plasma cholesterol (x) and the logarithmically transformed vessel wall cholesterol content (y). The linear regression expressed as the calculated correlation coefficient (r) was 0.88 with a slope of 0.037 for the placebo-treated animals. In this analysis, the mean vessel wall cholesterol value and the mean total plasma cholesterol exposure value of the control group were taken as the starting point of the regression. Using this strong linearity, we analyzed the effect of estradiol and tibolone treatment on the regression with the GLM procedure (SAS), in which the following model was used: yᵢⱼ=β₀+β₁xᵢ+εᵢⱼ; where i=treatment number (i=1 for placebo, i=2 for E₂-D, and i=3 for tibolone); j=animal number; yᵢⱼ=the log of the individual vessel wall cholesterol concentration of animal j on treatment i; xᵢ=the individual mean total plasma cholesterol exposure concentration of animal j on treatment i; β₁=the slope of the increase in the logarithmic vessel wall cholesterol level under treatment i; εᵢⱼ=random error of the measurement of animal j on treatment i; xᵢ=the mean log of vessel wall cholesterol level for the control animals; and xᵢ=the mean total plasma cholesterol level for the control animals. The calculated F values in the ANOVA were used for the P values of significance.
Body weight increased slightly but steadily during the experimental period, from \(\approx 2600\) to \(\approx 2900\) g. At week 20, no significant difference in body weight was found for the different treatment groups.

The cholesterol intake caused an increase in liver weight, from \(22\) g/kg body weight in the control group (group 1) to \(44\pm0.2\) g/kg body weight in the placebo group (group 2). Tibolone slightly but significantly inhibited this increase (\(32\pm0.1, 36\pm0.1,\) and \(39\pm0.1\) g/kg body weight for the 2-, 6-, and 18-mg dose, respectively). GPT concentrations increased slightly in the placebo group (from \(21.5\pm3.3\) to \(40.4\pm3.8\) U/L at week 20). The same was true for bilirubin, which increased from \(3.5\pm1.4\) to \(21.9\pm4.1\) \(\mu\)mol/L. These increases in the treatment groups were comparable or less than that in the placebo group.

The increase in body weight gain, the only slight increase in GPT concentrations, and the restricted increase in bilirubin concentrations indicate that the diet and treatments were, in general, tolerated well. However, from week 12 onward, 12 animals appeared to be high responders to the atherogenic diet. These animals became icteric and refused to eat. The animals were killed for ethical reasons (5 animals in group 1 and 2 in group 2 [placebo groups], 3 in group 4 [the E2-D group], 1 animal in group 5, and 1 animal in group 7). The number of animals in each group ending the study is presented in Table 1.

The OVX group fed the atherogenic diet (Pl_o; group 1) was taken as the reference group. The non-OVX group (group 2) showed no significant difference in any of the measured variables versus the OVX group. This finding confirms that in non-OVX rabbits, endogenous basal plasma estradiol concentrations are low (<7 pg/mL). For the sake of clarity, results from the non-OVX rabbits are not presented.

The atherogenic diet did not affect uterus weight, but uterus weight was increased in the E2, E2-D, E2-D/NETA, and tibolone-treated groups (Figure 1). The group treated with the highest dose of tibolone had a strong increase in uterus weight. The 2 lower-dose groups, however, showed increases in uterus weight comparable to or less than those in the E2-treated groups.

### Plasma Variables

**Plasma Hormone Concentrations**

Plasma estradiol concentrations were assessed during week 17 of treatment. Subcutaneous administration of E2-D gave fairly constant plasma concentrations over a period of 24 hours (63±5 pg/mL). Oral administration E2 showed peak concentrations after 1 hour of 238±23 pg/mL, which declined to 18±10 pg/mL after 24 hours. NETA did not affect plasma E2 concentrations. In the control OVX animals, estradiol concentrations were <2 pg/mL.

In humans, tibolone is rapidly metabolized into the 3α- and 3β-hydroxy metabolites, both with estrogenic properties, and the A4 isomer, which has progestogenic/androgenic properties. In rabbits, the same metabolites were found. For the 6-mg dose, the peak plasma concentrations of tibolone and its metabolites were in the same range as found in humans. The peak tibolone concentrations were 1.4±0.3 ng/mL 2 to 4 hours after oral administration, which is comparable to the concentrations found in humans (1.7 ng/mL). Eight hours after administration, the concentrations were below the detection limit of the assay.

**Plasma Lipids**

In the placebo OVX rabbits, the atherogenic diet caused a progressive increase in plasma cholesterol concentrations (Figure 2). After 20 weeks, plasma cholesterol concentrations were \(51\pm7\) mmol/L (mean±SE, \(n=9\)). In accord with other studies, lipoprotein analysis showed that the cholesterol increase was mainly due to an increase in the \(\beta\)-VLDL+IDL fraction. The HDL and LDL cholesterol fractions, after an initial increase, reached a plateau after 4 to 8 weeks. The mean plasma concentrations (calculated as the area under the curve of the plasma concentrations over 20 weeks divided by the duration of the experimental period in days) and the effects of the different treatments are shown in Table 2. This mean exposure value represents a more realistic average of cholesterol exposure to the arterial vessel wall during the experiment and corrects for the differences in shape of the cholesterol concentration curves over time. Neither sub-
Atheroprotective Effects of Tibolone in Rabbits

TABLE 2. Lipid Levels in the Study Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Plasma Triglycerides</th>
<th>Total Plasma Cholesterol</th>
<th>β-VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>HDL₂</th>
<th>HDL₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Pl₀</td>
<td>0.60±0.10</td>
<td>30.2±3.8</td>
<td>26.7±3.3</td>
<td>2.31±0.50</td>
<td>1.18±0.06</td>
<td>0.89±0.05</td>
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<td>2 Pl₉</td>
<td>0.72±0.12</td>
<td>30.6±2.5</td>
<td>27.6±2.4</td>
<td>1.90±0.23</td>
<td>1.17±0.05</td>
<td>0.86±0.05</td>
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</tr>
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<td>3 Ctrl</td>
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<td>1.0±0.1*</td>
<td>0.1±0.1*</td>
<td>0.22±0.04*</td>
<td>0.64±0.04*</td>
<td>0.42±0.04*</td>
<td>0.22±0.01*</td>
</tr>
<tr>
<td>4 E₂-D</td>
<td>0.42±0.03</td>
<td>34.3±2.8</td>
<td>30.7±0.1</td>
<td>2.27±0.04</td>
<td>1.28±0.09</td>
<td>0.95±0.02</td>
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<tr>
<td>5 E₂</td>
<td>0.49±0.04</td>
<td>30.4±3.7</td>
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<td>0.57±0.06</td>
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<td>1.77±0.33</td>
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<tr>
<td>7 NETA</td>
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<td>31.9±3.0</td>
<td>28.6±2.7</td>
<td>1.99±0.46</td>
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<td>0.40±0.03*</td>
<td>9.8±1.6*</td>
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<td>1.18±0.37*</td>
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<td>0.23±0.02*</td>
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<td>9 Tₐm</td>
<td>0.52±0.05</td>
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<td>1.06±0.27*</td>
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<td>1.04±0.11</td>
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<td>10 Tₘ</td>
<td>0.81±0.09*</td>
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<td>1.15±0.25*</td>
<td>1.29±0.10</td>
<td>1.03±0.10</td>
<td>0.26±0.02</td>
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</table>

Mean exposure per day is shown in mmol/L of total plasma triglycerides, total plasma cholesterol, and the cholesterol in plasma lipoproteins VLDL, LDL, HDL, HDL₂, and HDL₃ in the different treatment groups (see Table 1) induced by an atherogenic diet for 20 weeks. Values are mean±SE.

*P<0.05 compared with group 1.

Cutaneous nor oral treatment with estrogen (groups 4 and 5), NETA alone (group 7), and the E2/NETA combination (group 6) affected the mean plasma cholesterol or β-VLDL concentrations in comparison with placebo treatment. Tibolone attenuated the increase in total plasma cholesterol and β-VLDL concentrations at all doses. Tibolone did not affect the mean HDL and LDL exposure concentrations.

Plasma Triglycerides

Total plasma triglyceride concentrations were increased by the atherogenic diet. The concentrations were not clearly affected by the hormone treatment.

Arterial Vessel Wall

Cholesterol Accumulation in the Aorta

The atherogenic diet strongly increased aorta cholesterol concentrations. The accumulation, however, strongly differed in the different parts of the aorta. The increase was most pronounced in the aortic arch, reaching a concentration of 590±126 nmol/mg protein, followed by the abdominal aorta (172±31 nmol/mg protein) and thoracic aorta (71±11 nmol/mg protein). Figure 3 shows that E2-D caused an ≈50% reduction in cholesterol concentration in the 3 parts of the aorta. Orally administered E2, NETA, and the combination of E2/NETA in this respect were not effective. In the 3 tibolone-treated groups, however, the increase in vessel wall cholesterol was almost completely inhibited (inhibition of 97%). Compared with the OVX rabbits on a normal diet (control group), there was a slight but nonsignificant increase in cholesterol in the vessel wall.

To ascertain whether the effects of E2-D and tibolone on the arterial vessel wall were a function of plasma lipids, we applied regression analysis (see Figure 4). The placebo group consisted of groups 1 and 2, which were not significantly different from each other in mean total plasma cholesterol exposure (30.2±3.8 and 30.6±2.5 mmol/L, respectively) and vessel wall cholesterol concentration (590±126 and 630±132 nmol/mg protein, respectively). Linear regression analysis of the aortic accumulation of cholesterol in the vessel wall on the mean total plasma cholesterol exposure showed a strong correlation: a correlation coefficient of 0.88 and a slope of 0.037 for the placebo group. With this strong linearity, analysis of the combined data indicated that the 3 groups (placebo, E2-D, and tibolone) differed significantly from each other. E2-D showed a linear regression with a
correlation coefficient of 0.89 and a slope of 0.025, which was significantly different from the placebo group ($P < 0.0006$). For tibolone, the correlation coefficient was 0.38 and the slope was 0.010, which was significantly less steep than the slope of the placebo ($P < 0.0001$) and the E2-D ($P = 0.0006$) groups. By using the formula $y = ax + b$, the calculated vessel wall cholesterol concentration at a total plasma cholesterol exposure concentration of 20 mmol/L (a concentration around which plasma concentrations were found in all 3 groups) was 226 nmol/mg protein for the placebo group, 129 nmol/mg protein ($r = 0.89$) for the E2-D group, and to 65 nmol/mg protein ($r = 0.38$) for the 3 combined tibolone groups.

**Fatty Streak Formation in the Aorta**

Increased positive lipid staining was seen in all arterial segments of nontreated rabbits on the atherogenic diet. The sudanophilic surface coverage was 35% in the aortic arch, 2% in the thoracic aorta, and 9% in the abdominal aorta. The Sudan red–positive surface area in the animals on a normal diet was ,0.5%. Figure 5 shows that the only effective treatment in inhibiting fatty streak formation in the aortic arch was tibolone. E2-D did not affect fatty streak formation in the abdominal and thoracic aorta. E2, NETA, and the NETA combination with E2 did not significantly affect fatty streak formation.

**Vascular Reactivity**

Acetylcholine ($10^{-8}$ to $10^{-5}$ mol/L) concentration-dependently relaxed thoracic aortic rings of rabbits on a normal diet, which had been precontracted with 1 to $2 \times 10^{-7}$ mol/L phenylephrine ($\approx 50\%$ of the maximal contraction obtained with 50 mmol/L KCl). At $10^{-7}$ mol/L acetylcholine, the relaxation response was 35% of the maximal relaxation response. Aortic rings of placebo group rabbits on the atherogenic diet relaxed to only 4% at $10^{-7}$ mol/L acetylcho-

**Advanced Lesion Formation After De-endothelialization of the Carotid Artery**

De-endothelialization of the carotid artery induced intimal thickening at the site of de-endothelialization. In a few carotid arteries, however, occlusive lesions were formed. Histological examination showed that these occlusive lesions most probably were formed because of an occlusive thrombus immediately after the air-drying procedure (3 in the placebo group, 2 in 2 treatment groups, and 1 in each of the other 5 treatment groups). These animals were excluded from evaluation. De-endothelialization in combination with an athero-
tibolone. Tib indicates placebo (Pl-o). Testing of the control group with the tibolone groups showed a statistical difference, with \( P \) values of 0.03, 0.02, and 0.07 for the 18-, 6-, and 2-mg doses, respectively. Morphologically, the thickening had a compact structure and consisted mainly of circular smooth muscle cells (Figure 8C). Foam cells were virtually absent in the high- and medium-dose groups treated with tibolone. In the low-dose group, some foam cells were occasionally observed.

### Discussion

The results show that in rabbits, tibolone (at 2, 6, and 18 mg) has strong atheroprotective properties. In the arterial wall, tibolone almost completely prevented the processes of atherosclerotic lesion formation, such as accumulation of cholesterol, fatty streak formation, impairment of endothelium-dependent relaxation response, and advanced lesion formation after endothelial damage. Subcutaneously administered E2-D (150 \( \mu \)g) compared with tibolone showed a much less pronounced atheroprotective effect. E2-D inhibited cholesterol accumulation in the arterial to \( \approx \)50\%, whereas tibolone completely inhibited this increase even at the lowest dose tested (2 mg). E2-D strongly reduced fatty streak formation in the abdominal and thoracic part of the aorta. No effect, however, was observed on fatty streak formation in the aortic arch despite a 50\% reduction in cholesterol accumulation in this portion. This result indicates that E2-D in this section of aorta only reduced the thickening of fatty streaks. In contrast, tibolone completely inhibited fatty streak formation in the 3 portions of aorta. In a preliminary experiment (not shown), we used a 2\( \times \) higher dose of E2-D (300 \( \mu \)g per rabbit once weekly, \( n=13 \)) and, compared with the placebo group (\( n=22 \)), observed stronger atheroprotection than with the 150-\( \mu \)g dose but at the cost of a very strong (10-fold) increase in uterus weight. In this E2-D group, there was a reduction in cholesterol concentration of the aortic arch to 65\% (from 635\% to 224\% nmol/mg protein) and in fatty streak formation in the aortic arch to 60\% (from 42\% to 14\% coverage) compared with placebo. However, despite the high estrogen exposure resulting in a very strong stimulation of the uterus, the effect on the vessel wall was still significantly less than that obtained with the lowest dose of tibolone.

The difference in atheroprotective effect between tibolone and orally administered estradiol was even more pronounced than with E2-D (150 \( \mu \)g). The stronger effect of E2-D compared with orally administered E2 and the equipotent estrogen-mediated stimulation of the uterus suggest that the estrogen-induced effects on the arterial vessel wall are more pronounced with continuously elevated plasma estradiol concentrations than with estradiol concentrations that strongly vary over the course of the day. The peak plasma concentrations of E2 2 hours after oral administration were 238 pg/mL but declined to 18 pg/mL after 24 hours, whereas a once-weekly subcutaneous injection of E2-D resulted in constant plasma concentrations of \( \approx \)60 pg/mL over the course of the day. Our findings with oral estradiol alone and in combination with NETA (which did not affect the estrogen-induced
effects) are in accord with others, although we observed weaker effects. This finding was probably due to the longer duration of the atherogenic diet (20 weeks) and the higher cholesterol exposure in our model. The strong differences in atheroprotective properties of tibolone and estradiol cannot be explained by differences in estrogenic activity or the doses used.

Tibolone (at 2 and 6 mg) compared with oral E2, subcutaneous E2-D, and the estrogenic/progestogenic combination E2-D/NETA showed a similar or less pronounced increase in uterus weight, whereas the high dose of 18 mg tibolone showed a strong increase in uterus weight. Although tibolone has a hormonal profile different from that of estradiol, in the rabbit it is the estrogenic activity that induces the increase in

Figure 8. A representative transverse section (magnification ×426) of intimal thickening in an air-dried carotid artery of an OVX New Zealand White rabbit under the following conditions: fed an atherogenic diet and on placebo treatment (A). The intima consists of smooth muscle cells, foam cells (derived from smooth muscle cells and macrophages), and extracellular matrix; (B) fed an atherogenic diet and on E2-D treatment. The intima consists of smooth muscle cells, foam cells (mainly smooth muscle cells), and extracellular matrix; (C) fed an atherogenic diet and on tibolone (2-mg dose) treatment. The intima consists of smooth muscle cells and has a compact structure; and (D) fed a normal diet and on placebo treatment. The intima consists of smooth muscle cells and has a compact structure. I indicates intima; EM, extracellular matrix; L, lumen; M, macrophages; med, media; and SMC, smooth muscle cells.
uterus weight. No or very weak progestogenic activity was found on the endometrium (McPhail test in rabbits; Reference 52). Probably the estrogenic activity can counteract the progestogenic effect, a phenomenon common for estrogens in this test. This idea is confirmed by the finding that NETA did not modify the estrogenic effect of E2 on uterus weight (see Figure 1). This response is different from that in human females, in whom the hormonal profile of tibolone induces no or only weak endometrial stimulation, indicating that the estrogenic activity of tibolone on the endometrium is very weak.42,43

The doses used are based on human and preclinical studies (see Methods). In rabbits, tibolone is rapidly metabolized to the same metabolites as in humans: the 5α- and 3β-hydroxy metabolites, both with estrogenic properties, and the Δ4 keto isomer, with progestogenic/androgenic properties. This result was observed with the 6-mg dose in metabolite concentrations in plasma in the range of the concentrations observed in women and in a calculated estrogenic plasma activity comparable to the estradiol plasma concentrations obtained with E2-D. Thus, tibolone at doses of 2 and 6 mg, which induced plasma concentrations in the range of those observed in women, had equipotent estrogenic activity and induced an equipotent increase in uterus weight compared with E2-D, whereas the atheroprotective effect was much more pronounced. This suggests that tibolone, compared with E2-D, has tissue-selective properties.

An effect on plasma lipoprotein concentrations can contribute to the differences in atheroprotective properties of tibolone and E2-D. E2-D did not affect the diet-induced increases in total plasma cholesterol or cholesterol in the β-VLDL, LDL, and HDL lipoproteins. This indicates, in accord with previous findings,12–15 that the protective effect of estradiol on the arterial vessel wall is largely independent of plasma lipoprotein concentrations. In contrast, tibolone attenuated the diet-induced increase in plasma cholesterol concentrations. The tibolone-induced reduction in plasma cholesterol concentration was completely due to a reduction in β-VLDL cholesterol. How tibolone reduced plasma β-VLDL concentrations in the current study, however, is not clear and awaits further research. Despite the reduction in β-VLDL cholesterol, the observed plasma cholesterol concentrations should, owing to their long-lasting exposure to the vessel wall, lead to an increase in vessel wall cholesterol. It was observed by others13,14 that mean exposure concentrations of 8 to 12 mmol/L during 12 weeks resulted in an increase in vessel wall cholesterol from 50 to 150 nmol/mg protein. We found similar results (P.Z. et al, unpublished data, 1998). However, after tibolone treatment, almost no increase in vessel wall cholesterol was observed.

To ascertain whether the effect of tibolone on the arterial wall was a function of plasma lipids, we applied regression analysis. Linear regression analysis in the placebo group showed that there was a high correlation (correlation coefficient of 0.88) between plasma cholesterol concentration and vessel wall cholesterol concentration. With the use of this strong linearity, analysis of the combined data showed that the slopes of the regression lines for E2-D and tibolone were significantly less steep than that in the placebo group. The significantly reduced slope for E2-D indicates that E2-D reduces the accumulation of cholesterol in the vessel wall independently of an effect on plasma cholesterol. This notion is in accord with the literature14 and the current findings that E2-D reduced vessel wall cholesterol to ≈50% compared with the placebo-treated group without affecting plasma cholesterol concentrations. With tibolone, nearly no increase in slope was observed. The slope of tibolone also was significantly less steep than for E2-D. This indicates that not only estrogen but also tibolone (with an even more pronounced effect) protects the vessel wall from cholesterol accumulation independently of an effect on plasma cholesterol. Figure 4 shows that at a mean plasma cholesterol exposure of 20 mmol/L (a concentration around which plasma concentrations were found in all 3 groups), vessel wall cholesterol increased from 40 to 65, 129, and 226 nmol/mg protein for tibolone, E2-D, and placebo, respectively. However, although the strong reduction in accumulation of cholesterol in the vessel wall cannot be ascribed to plasma lipid or lipoprotein concentrations; for tibolone, the possibility cannot be excluded that the reduction in plasma β-VLDL concentrations can contribute to the strong atheroprotective effect and can partly explain the much more pronounced effect of tibolone on the arterial vessel wall compared with E2-D.

In rabbits on a high-cholesterol diet, β-VLDL becomes the main circulating lipoprotein while the increase in LDL and HDL reaches a plateau concentration, thus confirming results in the literature.12,14 This situation is different from that in the monkey model and in humans, in which LDL is the main circulating lipoprotein at much lower circulating cholesterol concentrations. The cellular processes leading to fatty streak formation, however, are comparable to those in humans. An increase in plasma β-VLDL induces an increase in vessel wall cholesterol (see also References 13 and 14). Although the LDL receptors are downregulated, the subendothelial cells are able to accumulate β-VLDL owing to the very high affinity of the apoE in β-VLDL for the LDL receptor. Therefore, increased plasma β-VLDL can contribute to cholesterol accumulation and fatty streak formation in the vessel wall.53,55 To exclude rabbit-specific effects and to investigate whether the effects of tibolone can be extrapolated to primates, the effect of tibolone in cynomolgus monkeys is currently under investigation.

One of the first indications of cholesterol accumulation in the vessel wall is the impaired endothelium-dependent relaxation response to acetylcholine (for a review, see Reference 54). Modified lipoproteins in particular seem to be responsible for the dysfunctionality of the endothelium. In vitro studies using isolated aortic rings with an intact endothelium showed that native LDL did not affect the endothelium-dependent relaxation response but that addition of modified LDL impaired the response.26 In the current study using aortic rings of rabbits fed a normal diet, acetylcholine caused a concentration-dependent relaxation, whereas aortic rings of rabbits on an atherogenic diet showed an impaired relaxation response. It has been shown that impairment of the response is due to a decreased release of NO from endothelial cells. NO synthesis itself, however, is not affected, as indicated by
the finding that the endothelium-dependent relaxation response to the calcium ionophore A23187 was unchanged (see also Reference 54). This outcome indicates that the atherosclerotic diet inhibits signal transduction from receptor to NO synthase in the endothelial cell. Estradiol is able to preserve the acetylcholine-induced relaxation response in the atherosclerotic vessel wall.55,56 In the current experiment, E2-D also inhibited the impairment of acetylcholine-induced relaxation. For tibolone, there was a dose-dependent effect. This profile is different from the effects on atherosclerotic lesion formation and suggests a dose-dependent estrogenic activity of tibolone on preservation of the relaxation response and the involvement of other factors in tibolone-induced atheroprotection.

Cholesterol accumulation, fatty streak formation, and impaired endothelium-dependent vasodilator responses are reversible processes but are the beginning of the formation of the irreversible advanced lesion.8,57 We mimicked the process of advanced lesion formation in the carotid artery of the rabbit by damaging the endothelial cell layer in combination with the atherogenic diet. Although E2-D inhibited advanced lesion formation, the lesions, however, still consisted of both smooth muscle cells and foam cells. The inhibitory effect of estradiol on smooth muscle cell proliferation is in accordance with findings that estradiol is able to inhibit the development of graft atherosclerosis in humans58 and smooth muscle cell proliferation of pig coronary arteries,59 rat carotid artery,60 and rabbit aorta and iliac artery61; to suppress surgically induced vascular intimal hyperplasia in rabbits62; and to inhibit proliferation in primary cultures of smooth muscle cells from rabbit aorta.63 In tibolone-treated animals the intimal thickening was, compared with placebo OVX animals, strongly inhibited and consisted mainly of smooth muscle cells (see Figure 7). Intimal thickening was even less than in rabbits on the normal diet. This suggests that tibolone also directly affects smooth muscle cell proliferation, (again) independent of plasma lipid concentrations. Thus, the plasma cholesterol–ol–independent effect of tibolone as observed via regression analysis on cholesterol accumulation is also observed in advanced lesion formation.

It has been suggested that advanced lesion formation in women mainly occurs after menopause. Therefore, it is possible that tibolone is not only effective in inhibiting fatty streak formation and restoring impaired vascular responses but also has the intrinsic potential to prevent the progression of advanced lesion formation in postmenopausal women.

Acknowledgments

We thank Willem Santegoets for expert technical work, Netty Lieberwirth for technical assistance and animal care, Pieternel van Heijst and Eugenie van den Berk for the plasma lipid assays, and Ad van Disseldorp for the histology work.

References

Atheroprotective Effects of Tibolone in Rabbits


Tibolone Prevents Atherosclerotic Lesion Formation in Cholesterol-Fed, Ovariectomized Rabbits

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Arterioscler Thromb Vasc Biol. 1998;18:1844-1854
doi: 10.1161/01.ATV.18.12.1844

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/18/12/1844

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