Progestogens Do Not Affect Aortic Accumulation of Cholesterol in Ovariectomized Cholesterol-Fed Rabbits

Jens Haarbo, Ole L. Svendsen, and Claus Christiansen

Cardiovascular disease is a major killer in postmenopausal women in the industrialized societies. To investigate the effect of progestogen and 17β-estradiol replacement therapy on atherogenesis, we studied 60 cholesterol-fed ovariectomized rabbits for 13 weeks. They were randomly assigned to four groups of 15 rabbits each and received oral treatment with norethisterone acetate, levonorgestrel, 17β-estradiol, or placebo. The active treatment groups achieved serum hormone concentrations, which produced physiological effects on the uterus. No significant differences in serum total cholesterol or ultracentrifuged lipoproteins (very low density, intermediate density, low density, or high density lipoproteins) were found between the four groups during the experimental period. There were no significant differences between the progestogen and placebo groups in the aortic accumulation of cholesterol. The estradiol group had only accumulated about half the aortic cholesterol as compared with the placebo group and the progestogen groups (p<0.05). The antiatherogenic effect of 17β-estradiol was estimated to be equal to a 40–50% reduction in serum total cholesterol. These findings suggest that two commonly prescribed 19-nortestosterone-derived progestogens, which are considered to be "atherogenic," do not affect atherogenesis in cholesterol-fed ovariectomized rabbits, whereas 17β-estradiol produces a significant antiatherogenic effect that is independent of lipid metabolism in plasma, possibly the result of a direct action on the arterial wall. (Circulation Research 1992;70:1198–1202)

Key Words • atherogenesis • aorta • lipids • sex hormones • rabbits

Cardiovascular diseases constitute a major health problem among postmenopausal women in the industrialized societies.1 Millions of these women receive hormone replacement therapy to alleviate climacteric complaints and prevent postmenopausal bone loss.2 In postmenopausal women with an intact uterus, a progestogen is often added to the estrogen therapy to negate the estrogen-mediated increase in endometrial cancer.2-3 Studies of lipid metabolism in postmenopausal women receiving estrogen-progesterogen replacement therapy4,5 have raised questions of vital importance: Are progestogens atherogenic? Do they reduce or eliminate the beneficial effect of estrogen monotherapy on cardiovascular disease?6-7 However, lipid metabolism in plasma is only one of many important determinants of atherosclerosis; therefore, it may be an inaccurate predictor of cardiovascular disease. This is in accordance with a study by the Lipid Research Clinic, which found that only 50% of the beneficial effect of estrogens was explained by the influence on serum lipids and lipoproteins.6 In addition, recent experiments with different animal models indicate that progestogens in combination with estrogen do not attenuate the beneficial estrogenic effect.8-10 To study further the independent impact of progestogens on atherogenesis, we investigated the effect of norethisterone acetate and levonorgestrel (derivatives of 19-nortestosterone and considered "atherogenic" progestogens) on aortic accumulation of cholesterol in a prospective randomized and controlled study of cholesterol-fed ovariectomized rabbits with a positive (estrogen monotherapy) and a negative (placebo) control group.

Materials and Methods

Sixty sexually mature female white rabbits, weighing 2.7–4.4 kg, of the Danish Country strain were purchased from Statens Serum Institute. They were housed individually at the Panum Institute, University of Copenhagen, in stainless-steel cages at a room temperature of 20±2°C, at 50±10% humidity, and with a 12-hour light cycle. The study ran for 13 weeks.

In week 1, the rabbits were anesthetized and underwent a bilateral ovariectomy.9 In week 2, they were randomized to four groups of 15 rabbits each and received oral treatment with 1 mg norethisterone acetate (NETA group), 0.5 mg levonorgestrel (LNG group), 4 mg 17β-estradiol (E2 group), or no hormones (placebo group). All rabbits were fed 80 g of chow daily, which consisted of hormones (Schering AG, Berlin) or placebo, 6.4 g corn oil (Mecobenzon, Copenhagen), 0.32 g cholesterol (USP, Sigma Chemical Co., St. Louis, Mo.), and standard rabbit pellets (Superfoss, Den-
TABLE 1. Baseline Characteristics, Total Food Intake, and Body Weight Gain in Treated and Untreated Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>NETA</th>
<th>LNG</th>
<th>E2</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>20.3±1.6</td>
<td>19.5±2.1</td>
<td>19.4±1.6</td>
<td>20.7±2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>3.6±0.5</td>
<td>3.5±0.5</td>
<td>3.4±0.4</td>
<td>3.3±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>1.7±0.8</td>
<td>1.7±0.8</td>
<td>1.8±1.1</td>
<td>1.6±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>TGs (mmol/l)</td>
<td>0.7±0.3</td>
<td>0.9±0.4</td>
<td>0.9±0.5</td>
<td>1.1±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>0.6±0.2</td>
<td>0.6±0.1</td>
<td>0.5±0.2</td>
<td>0.6±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight gain (kg)</td>
<td>0.2±0.4</td>
<td>0.3±0.3</td>
<td>0.2±0.3</td>
<td>0.2±0.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SD. Placebo, rabbits treated with no hormones; NETA, rabbits treated with 1 mg norethisterone acetate; LNG, rabbits treated with 0.5 mg levonorgestrel; E2, rabbits treated with 4 mg 17β-estradiol; ANOVA, analysis of variance; TC, total cholesterol; TGs, triglycerides; HDL-C, high density lipoprotein cholesterol; NS, not significant.

mark), as described in detail elsewhere. The rabbits had free access to water.

Blood samples were taken 24 hours after the last feeding. Serum total cholesterol and triglycerides were determined by an enzymatic method (Chem I, Technicon Instruments Corp., Tarrytown, N.Y.) before the ovariectomy, in weeks 4, 6, 8, 10, and 12, and at the end of week 13. High density lipoprotein cholesterol was initially measured after precipitation with phosphotungstate-MgCl2. In addition, three aliquots from the serum samples collected at weeks 8 and 12 were adjusted sequentially to a density of 1.006, 1.019, and 1.063 g/ml, respectively, and centrifuged at 4°C at 1.58×106 g·min in a 50.4 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). Top and bottom fractions were obtained after tube slicing. The cholesterol content in the aliquots of whole serum and the ultracentrifuged fractions was determined by an enzymatic method.

Serum concentrations of norethisterone acetate, levonorgestrel, and estradiol were measured by radioimmunoassays as described elsewhere.

At the end of the 13-week experiment, the rabbits were anesthetized with intravenous injections of a 5% pentobarbital solution. The thoracic aorta was dissected free, and the adventitia was carefully removed under running saline. The aorta was opened longitudinally, and the surface was rinsed with saline. The proximal part (above the first intercostal artery) was fixed with pins on a corkboard. Thereafter, the inner layer containing the intima and part of the media was stripped from the underlying outer media and weighed. The tissue was immediately stored at −20°C until analyzed.

The aortic tissue (the inner layer of the proximal part) was minced, and the lipids were extracted with chloroform and methanol (2:1 vol/vol) over 24 hours. Lipids and proteins were separated. The total cholesterol content was determined enzymatically after evaporation of the fraction containing cholesterol and dissolution in isopropyl alcohol. The total cholesterol content was adjusted for the amount of protein in the tissue specimens, which was determined by the method of Lowry et al after extraction of the lipids and digestion of the residue for 24 hours with 5 M NaOH. To compare this new method with the traditional Liebermann-Burchard test, we measured 74 liver samples from an earlier study by both methods. After logarithmic transformation to normalize variations, the coefficient of correlation was 0.96, and the intercept and slope were not significantly different from 0 and 1, respectively. The accuracy error (SEE) and the intersection imprecision were 3.2% and 2.9%, respectively.

The endometrial activity of estradioldehydrogenase was measured as previously described. The protein content of the endometrial tissues was determined by the method of Lowry et al.

Statistics


Analysis of variance (ANOVA) was used to compare food intake, gain in body weight, serum lipids and lipoproteins, activities of endometrial estradioldehydrogenase, serum hormone concentrations, and aortic accumulation of cholesterol. The effect of estradiol therapy and serum total cholesterol on aortic accumulation of cholesterol was analyzed by a separate-slopes model with the GLM procedure (SAS) (Figure 2). The data on aortic cholesterol and endometrial estradioldehydrogenase activity were logarithmically transformed to normalize variations.

Results

The groups were well matched with regard to age, body weight, and initial serum lipids and lipoproteins (Table 1). One rabbit (NETA group) had to be killed in week 2 because of a broken back; thus, 59 completed the study. There were no visible side effects on the general condition or behavior during the experimental period. Furthermore, all four groups ate more than 96% of the offered chow, and they did not show significant differences in body weight gain (Table 1).

The mean serum concentration of total cholesterol and triglycerides (based on seven measurements during the experimental period) are given in Table 2. Serum total cholesterol tended to be higher in the LNG group than in the other three groups; however, this was not of statistical significance. Serum triglycerides were higher in the LNG group than in the other groups (p<0.001), but this parameter was not statistically significantly related to aortic accumulation of cholesterol. Table 2 also shows the mean distribution of cholesterol over the different lipoproteins (in percentage of serum total cholesterol) as measured in weeks 8 and 12. No significant differences were found in these parameters or in the total cholesterol/high density lipoprotein cholesterol ratio.

Table 3 shows the fasting serum hormone concentrations, the chow-mediated increase in these parameters, and the activity of the endometrial estradioldehydroge-nase adjusted for protein content. The fasting concentration and the chow-mediated increase in the serum
estradiol concentration were significantly higher in the E2 group than in the other three groups (p<0.0001). In addition, the placebo group had the lowest fasting serum concentration of estradiol. Serum concentrations of progestogens were below the detection level in the E2 and placebo groups. The endometrial estradioldehydrogenase activity was significantly higher in the E2 group than in the placebo group (p<0.0001), which again was higher than in the progestogen groups combined (p<0.05).

Figure 1 visualizes the aortic content of cholesterol and shows that the E2 group had significantly lower aortic accumulation of cholesterol than the other groups (p<0.05 by ANOVA). There were no significant differences among the placebo group and the progestogen groups. Adjustment for mean serum total cholesterol or lipoprotein variables using analysis of covariance did not affect these results (0.005<p<0.05). The risk of overlooking a difference of 25 nmol/mg (20%) in aortic accumulation of cholesterol between the progestogen and placebo groups was estimated in the present study to be less than 15%.

Figure 2 visualizes the regressions of aortic accumulation of cholesterol on the mean serum total cholesterol in the placebo and E2 groups. The intercepts were statistically significantly different (p<0.01), whereas the slopes were identical. As visualized in the figure, this difference means that the value of the estradiol-mediated antiatherogenic effect, which is independent of serum total cholesterol, corresponds to a significant (40–50%) reduction in serum total cholesterol.

### Discussion

The principal findings in the present study were that two progestogens (norethisterone acetate and levonorgestrel) believed to be atherogenic do not affect the aortic accumulation of cholesterol significantly and that 17β-estradiol has a significant beneficial effect on atherogenesis that is independent of serum lipids and lipoproteins in cholesterol-fed ovariectomized rabbits. Studies in monkeys and rabbits indicate that the addition of progestogens does not reduce the antiatherogenic effect of estradiol.8,9 In these studies, the progestogens were given in doses that achieved relevant serum concentrations and negated the estradiol-mediated increase in high density lipoprotein cholesterol. The present results extend those observations and indicate that progestogens have a neutral effect on atherogenesis. Extrapolation of results obtained from cholesterol-fed rabbits to humans should always be done cautiously because of the differences in lipoprotein metabolism in plasma. For instance, the neutral progestogen effect on high density lipoprotein cholesterol in the present study does not allow us to exclude a progestogenic influence on atherogenesis mediated through this parameter in humans. However, the present data support the view that even the most atherogenic progestogens can be combined with estradiol therapy to protect postmenopausal women against endometrial cancer without increasing their risk of cardiovascular disease. In addition, the progestogens may have an independent beneficial effect on bone metabolism.17

### Table 2. Mean Serum Concentration of Total Cholesterol and Triglycerides and Mean Distribution of Total Cholesterol Over the Lipoproteins in Treated and Untreated Rabbits

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Placebo</th>
<th>NETA</th>
<th>LNG</th>
<th>E2</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean TC (mmol/l)</td>
<td>27.3±3.2</td>
<td>24.9±2.1</td>
<td>31.2±3.3</td>
<td>25.7±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Mean TGs (mmol/l)</td>
<td>1.18±0.1</td>
<td>0.99±0.1</td>
<td>2.74±0.5</td>
<td>0.86±0.1</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>VLDL-C (%)</td>
<td>49.5±2.7</td>
<td>45.6±3.3</td>
<td>53.0±4.2</td>
<td>43.5±2.8</td>
<td>NS</td>
</tr>
<tr>
<td>IDL-C (%)</td>
<td>24.5±3.2</td>
<td>24.6±1.4</td>
<td>21.7±1.7</td>
<td>28.8±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (%)</td>
<td>21.0±3.2</td>
<td>24.2±2.9</td>
<td>21.6±2.6</td>
<td>24.4±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (%)</td>
<td>5.0±1.0</td>
<td>5.7±1.4</td>
<td>3.6±0.8</td>
<td>4.3±1.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Placebo, rabbits treated with no hormones; NETA, rabbits treated with 1 mg norethisterone acetate; LNG, rabbits treated with 0.5 mg levonorgestrel; E2, rabbits treated with 4 mg 17β-estradiol; ANOVA, analysis of variance; TC, total cholesterol; TGs, triglycerides; VLDL-C, very low density lipoprotein cholesterol; IDL-C, intermediate density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; NS, not significant. Mean serum concentrations of TC and TGs are based on seven measurements. Mean distribution of TC over the lipoproteins was measured in weeks 8 and 12 and is given as percent of TC.

### Table 3. Mean Hormone Levels in Treated and Untreated Rabbits

<table>
<thead>
<tr>
<th>Progestogen</th>
<th>Placebo</th>
<th>NETA</th>
<th>LNG</th>
<th>E2</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (pmol/l)</td>
<td>47±3</td>
<td>76±6</td>
<td>93±6</td>
<td>167±19</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>ΔEstradiol (pmol/l)</td>
<td>41±10</td>
<td>58±9</td>
<td>-17±13</td>
<td>659±146</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Progestogen (nmol/l)</td>
<td>&lt;0.3</td>
<td>0.9±0.1</td>
<td>0.8±0.1</td>
<td>&lt;0.3</td>
<td>...</td>
</tr>
<tr>
<td>ΔProgestogen (nmol/l)</td>
<td>&lt;0.3</td>
<td>8.4±3.0</td>
<td>4.4±0.5</td>
<td>&lt;0.3</td>
<td>...</td>
</tr>
<tr>
<td>EDH (nmol E2/mg protein per hour)</td>
<td>0.5±0.1</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
<td>7.2±3.2</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Placebo, rabbits treated with no hormones; NETA, rabbits treated with 1 mg norethisterone acetate; LNG, rabbits treated with 0.5 mg levonorgestrel; E2, rabbits treated with 4 mg 17β-estradiol; ANOVA, analysis of variance; Δ, change from before feeding to 3 hours after feeding in week 14; EDH, estradioldehydrogenase activity; E2, estrone. Mean hormone levels were measured in all rabbits in weeks 10 and 14; change (Δ) was measured in seven randomly selected rabbits from each of the four groups.
FIGURE 1. Graph showing the aortic accumulation of cholesterol in the four groups: placebo, rabbits treated with no hormones; NETA, rabbits treated with 1 mg norethisterone acetate; LNG, rabbits treated with 0.5 mg levonorgestrel; E₂, rabbits treated with 4 mg 17β-estradiol. Analysis of variance (ANOVA) indicated significant differences between groups, and Student’s t test showed that the E₂ group had a significantly lower aortic accumulation of cholesterol than any of the other three groups.

Whether other progestogens considered to be less atherogenic, e.g., 17-hydroxyprogesterone derivatives, might have an independent protective effect on atherogenesis is largely unknown. A study in which progesterone was administered parenterally did not demonstrate an independent effect on atherogenesis in ovariectomized rabbits. The suggestion that 17-hydroxyprogesterone and 19-nortestosterone derivatives have comparable effects on atherogenesis is borne out by a recent report in which equipotent doses of the substances had similar overall effects on serum lipids, lipoproteins, and apolipoproteins. These studies may therefore suggest that in equipotent doses progestogens have equal effects on atherogenesis and that their postulated differences are overestimated.

Contrary to our previous study, the present experiment did not show a significant lowering effect of estradiol on serum total cholesterol (very low density lipoprotein cholesterol and intermediate density lipoprotein cholesterol). This discrepancy may at least partly be explained by different study designs. The rabbits of the present study were throughout the (shorter) experimental period fed a high-cholesterol diet, whereas our previous study included an initial period with low-cholesterol feeding and hormone treatment. These findings suggest that the hormonal effect on the lipid metabolism in plasma (for instance, on the low density lipoprotein receptor) in cholesterol-fed rabbits may depend on the absolute serum concentrations of lipids.

The present study demonstrates that 17β-estradiol possesses a protective effect against aortic accumulation of cholesterol, which is consistent with previous findings in humans and experimental animals. The effect was not related to the serum concentrations of lipids and lipoproteins, and its magnitude was comparable to findings in our previous study, in which the same doses were used. This may be a direct effect of estradiol on the arterial wall, which is consistent with a recent preliminary report showing that 17β-estradiol cyclically combined with progestosterone significantly reduces the accumulation of radioactively labeled low density lipoprotein cholesterol in the aorta of ovariectomized monkeys. This effect was independent of other morphological parameters and was supported by another preliminary study in rabbits. The E₂ group accumulated only about half the aortic cholesterol as compared with the placebo group. This difference was estimated to equal a 40–50% reduction in serum total cholesterol. Although generalization from experimental animals should be done with caution, these results fit theoretically with recent epidemiological studies showing a significant reduction in cardiovascular and overall mortality in women. Thus, the present data support the conclusion of a recent review article, namely, that estrogen replacement ought to be considered as prophylactic therapy in postmenopausal women at risk of heart disease, which, when present, may be more dangerous for women than men.

The oral hormone doses administered in the present study were, on a per-weight basis, rather large. Nevertheless, no signs of a toxic reaction were seen on body weight gain, food intake, or general well-being. The serum concentrations obtained, which presumably more closely reflect the physiological response in the arterial wall, were comparable to the concentrations found in postmenopausal women receiving hormone replacement therapy. The endometrial response is further evidence that the doses used in the present study produced physiological effects.

Aortic cholesterol (nmol/mg protein)

FIGURE 2. Graph showing the relation (and coefficient of correlation r) between aortic accumulation of cholesterol and the mean serum total cholesterol concentration based on seven measurements during the experimental period in the estradiol group (●) and the placebo group (○). The intercepts (β) were significantly different in the two groups (βestradiol = 3.37, and βplacebo = 3.80; p < 0.05), whereas the slopes (α) were identical (αestradiol = 0.032, and αplacebo = 0.037). Log aortic cholesterol is presented as α × (mean serum total cholesterol) + β. The figure shows that the estradiol and placebo groups accumulated the same amount of aortic cholesterol at serum concentrations of 35 and 20 mmol/l, respectively.
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Progestogens do not affect aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits.
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