Raloxifene and estrogen reduces progression of advanced atherosclerosis — a study in ovariectomized, cholesterol-fed rabbits

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Abstract

The present study investigated the effect of raloxifene, a selective estrogen receptor modulator (SERM), on aortic atherosclerosis in 80 ovariectomized, cholesterol-fed rabbits with pre-induced atherosclerosis. The animals were fed an atherogenic diet containing 240 mg cholesterol/day for 15 weeks, after this period a baseline control group was sacrificed. Thereafter, oral treatment was initiated with either estradiol 4 mg/day (n = 20), raloxifene (210 mg/day) or placebo (n = 20). In the treatment period of 39 weeks, the dietary cholesterol content was reduced to 80 mg cholesterol/day. Postmortem evaluation showed a significantly increased uterine weight induced by estradiol treatment (10.3 ± 1.2 g), whereas raloxifene intervention caused a decreased uterus weight (1.21 ± 0.1 g) when compared to placebo (2.48 ± 0.47 g). Throughout the study, serum lipids increased in all groups to levels seen in very high risk humans. After 58 weeks the cholesterol content in the aorta was 3.18 ± 0.54 mmol/cm² (38% reduction) in the estradiol group, 3.66 ± 0.52 mmol/cm² (29% reduction) in the raloxifene group and 5.12 ± 0.60 mmol/cm² in the placebo group. Analyses of the aortic cholesterol content corrected for time-averaged serum cholesterol revealed that both estradiol and raloxifene therapy significantly reduced the progression of atherosclerosis (P < 0.01 for both) as compared to placebo. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atherosclerosis; Lipids; Cholesterol; Prevention; Estrogen; Raloxifene; Endometrium

1. Introduction

The prevalence, morbidity and mortality of cardiovascular disease in postmenopausal women requires a concerted investigational effort to define efficacious prevention strategies. The increase in cardiovascular events with age in elderly women has been related to the decrease in endogenous estrogen production after menopause [1], and numerous observational data have shown a protective effect of estrogen treatment, particularly in secondary prevention [2–4].

The recent null conclusion of the Heart and Estrogen/progestin Replacement Study (HERS) [5] has raised doubts about the exact effect of female hormones upon ischemic heart disease and the side effect profile of traditional HRT doses prevents many women from receiving long-term treatment [6]. Raloxifene, a selective estrogen receptor modulator (SERM), is distinguished from estrogen by its ability to interact with the estrogen receptor either as an estrogen agonist or antagonist depending on the target tissue and hormonal milieu [7]. Thus, raloxifene has estrogen-like effects upon bone [8,9] but anti-estrogenic effects on breast [10] and endometrium [8,9]. The influence of raloxifene on cardiovascular risk markers has demonstrated both similarities and differences from that of estrogen [11].

It has previously been shown that both estradiol and raloxifene treatments inhibit atherosclerosis formation in healthy rabbit arteries [12–14]. In rabbits with pre-induced formation of atheroma, estradiol inhibited fur-
ther progression of atherosclerosis [15,16]. Thus, it was important also to study the effect of raloxifene in rabbits with pre-induced formation of atheroma, which may mimic mild atherosclerosis as seen in early post-menopausal women.

2. Experimental animals and methods

2.1. Experimental animals

Eighty healthy and sexually mature female rabbits of the Danish Country strain (SSC: CPH), were obtained at Statens Serum Institut, Copenhagen, DK. The rabbits, which previously had been fed a standard commercial rabbit chow, were housed at approved animal facilities (Ledoeje, DK) in standard rabbit cages with a room temperature of 20 ± 2°C and a 12-h light cycle. All rabbits had free access to water and were administered 100 g of chow/day at 08:00 h. The study was approved by the Danish authorities for experimental animals (Department of Justice). Body weight was checked every 4 weeks on the same scale. The chow containing estradiol, raloxifene or placebo was prepared, stored and controlled as previously described [13,14].

2.2. Study design

The rabbits (n = 80) were allowed to acclimate for 3 weeks, were ovarioctomized the following week as described earlier [13,14] and randomised to either baseline control (n = 20), placebo (n = 20), estradiol 4 mg/day (n = 20) or raloxifene 210 mg/day (n = 20). In week 5 the induction of atherosclerosis was initiated by including 240 mg cholesterol/day in the diet. In week 19 the baseline control group was sacrificed to measure the baseline level of atherosclerosis. To stabilize serum lipids, the amount of cholesterol in the diet was reduced to 80 mg/day from week 20–58. In the same time period, the rabbits were treated orally as indicated by randomisation. Blood sampling was performed in weeks 13, 19, 27, 42 and 57, approximately 24 h after the last feed.

2.3. Biochemistry

Serum total cholesterol, high density lipoprotein (HDL)-cholesterol (only at baseline) and triglycerides were determined enzymatically by routine analysis procedures. Serum lipoproteins were fractionated into HDL (density > 1.063 g/ml), low density lipoprotein (LDL) (1.019 g/ml < density < 1.063 g/ml), intermediate density lipoprotein (IDL) (1.006 g/ml < density < 1.019 g/ml) and very low density lipoprotein (VLDL) (density < 1.006 g/ml) by ultracentrifugation and measured in weeks 13, 19, 27, 42 and 57 as previously described [13,14].

2.4. Aortic tissue

The thoracic aorta was freed from the adventitia, opened longitudinally and divided into proximal and distal parts at the level of the first intercostal arteries. After determination of the surface area of the proximal part, the inner layer containing the intima and part of the media was stripped from the underlying outer media. The inner layer, which was used for the assessment, was weighed and stored at −20°C. The total cholesterol and protein content was then determined according to published procedures [13].

2.5. Uterus and endometrial tissue

The bicorn uterus of the rabbits was cut at the bifurcation, weighed and opened. A sample of endometrial tissue was excised from the cavity, frozen immediately on solid carbon dioxide and stored at −85°C. For the analyses the tissue was homogenised and a cytosolic fraction and a nuclear extract were prepared as previously described [13]. The estrogen receptor content in the cytosolic fraction and in the nuclear extract was measured by an enzyme immunoassay according to the manufacturer’s instruction (Abbott Laboratories, North Chicago, IL). The cytosolic progesterone binding capacity was measured by a steroid binding assay using dextran-coated charcoal separation [17]. The results of the receptor contents were then normalised to the cytosolic protein content, which was determined as previously [13] according to Bradford.

2.6. Statistics

All analyses were performed using Statistical Analysing System (SAS). The time averaged concentration of serum lipids and lipoproteins was calculated as the area under the curve divided by the duration of the study period in weeks. One-way analysis of variance (ANOVA) was used to test for significant differences between the four groups with regard to baseline age, body weight, serum lipids and lipoproteins. ANOVA was furthermore used to compare food consumption, uterus weight, endometrial estrogen receptor content and cholesterol content of aorta. If the tests revealed significant differences, Student’s t-test was used for the group-by-group comparison. A multiple linear regression model (GLM procedure) was used to examine the independent effects of treatment and time averaged lipids and lipoproteins on the aortic accumulation of cholesterol.
cholesterol content for the baseline sacrifice group were proteins throughout the study (Tables 1 and 2). The regard to food intake, weight gain, lipids and lipoproteins were seen (Table 1). Three rabbits died prematurely, two in the estradiol group and one in the placebo group, thus 77 rabbits completed the study. The groups were comparable with respect to age, body weight, lipids or lipoproteins (Table 1). The estradiol group had 24.6 ± 9 mmol, 5.12 ± 0.10 mmol, and 42.0 ± 6.4 mmol of cholesterol, triglycerides, whereas this was not the case for LDL-cholesterol and HDL-cholesterol. After correction for these values of lipids and lipoproteins, an independent effect of treatment upon aortic atherosclerosis was demonstrated and Fig. 1A and B show the results corrected for serum cholesterol. For area-adjusted aortic cholesterol content, the response in the estradiol group was 62% of that of placebo, whereas for raloxifene the response was 71% of placebo. The aortic cholesterol content adjusted for protein showed comparable responses, although only of borderline significance.

The uterine wet weight was significantly increased due to estradiol treatment (10.3 ± 1.2 g; P < 0.001), in contrast to raloxifene therapy, which significantly reduced the weight of the uterus (1.21 ± 0.1 g, P < 0.05) as compared to placebo (2.48 ± 0.47 g) (Fig. 2A). The endometrial nuclear estrogen receptor content was up-regulated in the raloxifene group (P = 0.02), whereas estradiol therapy tended to decrease the number of estrogen receptors in the nucleus (P = 0.16) (Fig. 2C). In the cytosol of endometrial cells, both estradiol and raloxifene downregulated estrogen receptors (P < 0.001 for both) (Fig. 2D), whereas progesterone receptors were increased in the estradiol group (P = 0.02) and

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Raloxifene (N = 20)</th>
<th>Estradiol (N = 18)</th>
<th>Placebo (N = 19)</th>
<th>Control (N = 20)</th>
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<tr>
<td>Age (months)</td>
<td>6.5 ± 0.4</td>
<td>6.4 ± 0.3</td>
<td>6.3 ± 0.4</td>
<td>6.2 ± 0.4</td>
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<tr>
<td>Body weight (kg)</td>
<td>3.47 ± 0.28</td>
<td>3.65 ± 0.54</td>
<td>3.57 ± 0.32</td>
<td>3.55 ± 0.30</td>
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<tr>
<td>S-total cholesterol (mmol/l)</td>
<td>1.77 ± 0.53</td>
<td>1.70 ± 0.56</td>
<td>1.81 ± 0.35</td>
<td>1.58 ± 0.44</td>
</tr>
<tr>
<td>S-HDL-cholesterol (mmol/l)</td>
<td>0.75 ± 0.16</td>
<td>0.63 ± 0.18</td>
<td>0.72 ± 0.22</td>
<td>0.66 ± 0.19</td>
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<tr>
<td>S-triglycerides (mmol/l)</td>
<td>0.58 ± 0.19</td>
<td>0.64 ± 0.27</td>
<td>0.67 ± 0.21</td>
<td>0.59 ± 0.30</td>
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<tr>
<td>Body weight gain (kg)</td>
<td>0.76 ± 0.06</td>
<td>0.87 ± 0.09</td>
<td>0.88 ± 0.05</td>
<td>–</td>
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<tr>
<td>Total food intake (kg)</td>
<td>37.8 ± 0.0</td>
<td>37.6 ± 0.1</td>
<td>37.8 ± 0.0</td>
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</tr>
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* ANOVA: all are NS.

### Table 2

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<th>Estradiol (N = 18)</th>
<th>Placebo (N = 19)</th>
<th>P, ANOVA</th>
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<td>S-total cholesterol (mmol/l)</td>
<td>17.41 ± 1.30</td>
<td>16.84 ± 1.79</td>
<td>16.57 ± 1.28</td>
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<td>S-VLDL-cholesterol (mmol/l)</td>
<td>11.10 ± 1.08</td>
<td>10.12 ± 1.33</td>
<td>9.27 ± 1.05</td>
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<td>S-LDL-cholesterol (mmol/l)</td>
<td>3.82 ± 0.55</td>
<td>5.64 ± 0.60</td>
<td>4.61 ± 0.47</td>
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<td>S-IDL-cholesterol (mmol/l)</td>
<td>5.26 ± 0.37</td>
<td>5.02 ± 0.45</td>
<td>4.63 ± 0.41</td>
<td>NS</td>
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<td>S-HDL-cholesterol (mmol/l)</td>
<td>1.63 ± 0.67</td>
<td>0.50 ± 0.03</td>
<td>0.94 ± 0.30</td>
<td>NS</td>
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<td>S-triglycerides (mmol/l)</td>
<td>0.92 ± 0.07</td>
<td>0.89 ± 0.11</td>
<td>1.06 ± 0.14</td>
<td>NS</td>
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</table>
borderline increased in the raloxifene group ($P = 0.16$) (Fig. 2B).

4. Discussion

The data show a reduction in atherosclerosis progression on atherosclerotic arteries during treatment with raloxifene and estradiol in cholesterol-fed, ovariec-
tomized rabbits. This effect is only partially explained by changes in serum lipids and lipoproteins.

The study was designed to evaluate the effect of raloxifene upon pre-existing atherosclerosis, therefore the baseline sacrifice group was added to assess the degree of atherosclerosis at the time of initiation of treatment and included placebo and estradiol groups as negative and positive controls.

A 38% reduction in atherosclerosis formation due to estradiol treatment was comparable to previous secondary prevention studies, which ranged from 24 [16] to 53% [15]. The somewhat milder effect of raloxifene (29% reduction) was also comparable to results from primary prevention experiments in rabbits, where a 30% reduction was found on the same raloxifene dose [14]. This dose was chosen to produce plasma raloxifene levels comparable to those of postmenopausal women during raloxifene therapy [18]. Table 3 shows the accumulated results in the rabbit model of estradiol and raloxifene, respectively. These data show a tendency for a less pronounced response when atherosclerosis is present at the initiation of therapy (secondary prevention model).

A recent study of conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA), involving 2700 women with documented, advanced coronary heart disease, failed to demonstrate preventive effects upon myocardial infarction and cardiac death. In fact, in the first 2 years of the study an increased risk during active treatment was seen whereas the risk decreased during the third and fourth year [5]. Theoretically, this may be due to an initial negative effect upon haemosta-
sis, while the effect upon atherosclerotic plaque formation and stability becomes evident thereafter [5]. These results may cast doubt on the value of CEE + MPA upon secondary prevention, but not necessarily on the potential for raloxifene, although both treatment regi-
mens have been found to increase the risk of venous thrombosis with a relative risk of 3 [5,10]. Although the current consensus based on observational data is that HRT protects against atherosclerosis, these results are
likely to be biased because of baseline differences between women who chose — or chose not — to receive long-term treatment with estrogens. Thus, the effect of HRT in general may be significantly less than originally assumed, but it may also be that only certain populations will benefit or that only particular regimes may have a clinically relevant efficacy.

The effect of raloxifene on CVD is not fully elucidated but in postmenopausal women, raloxifene decreases serum total and LDL-cholesterol, and has a neutral effect on triglycerides and HDL-cholesterol [9,11]. Raloxifene also decreases plasma fibrinogen, but has no effect on markers of fibrinolysis. This was in contrast to conjugated equine estrogens co-administered with medroxyprogesterone acetate (CEE + MPA), which had a positive effect on fibrinolysis markers, but no influence on coagulation markers [11]. As suggested by the data, non-lipid mechanisms may be involved in a possible beneficial effect of raloxifene. This is supported by recent data showing that raloxifene relaxes rabbit and human coronary arteries acutely by an endothelial-dependent mechanism involving NO [19,20]. Results from a study on levormeloxifene and estradiol furthermore indicate a role for NO in antiatherogenic effects of estrogen [21]. Taken together, these data may provide a rationale for a hypothesis on a non-lipid mechanism of action.

A possible cardioprotective effect of raloxifene in postmenopausal women will be investigated in the RUTH trial, a study in 10 000 women at risk for ischemic heart disease, which is currently in its enrolment phase. The study is powered to show a 17.5% reduction in cardiac events due to raloxifene intervention and excludes women with increased risk for venous thrombosis and expected to show results in 2005.

The partially estrogenic and non-estrogen effects of raloxifene were confirmed by the uterine data, which elegantly demonstrated that raloxifene exerts both estrogen agonist and estrogen antagonist effects in the same target organ in rabbits. Thus, whereas raloxifene in contrast to estradiol decreases uterine weight and upregulates the nuclear content of estradiol receptors, both therapies increase cytosolic progesterone receptors and decrease cytosolic estrogen receptors. In the MORE trial, a study in 7700 osteoporotic women, raloxifene did not increase the risk of either endometrial hyperplasia or uterine cancer [10].

In conclusion, in a series of three experiments investigating the effect of raloxifene on atherosclerosis in ovariectomized, cholesterol-fed rabbits a 30% reduction has consistently been shown in aortic cholesterol content, which is only partially explained by lipid lowering. While awaiting the results from the RUTH study, possible mechanisms for non-lipid actions of raloxifene should be further studied.

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References


