Abstract 17β-Estradiol has recently been found to inhibit atherogenesis by mechanisms that are in part independent of the estrogenic action on plasma lipoprotein levels. Since aortic permeability to low-density lipoprotein (LDL) in normocholesterolemic rabbits is a strong predictor for subsequent atherosclerosis during hypercholesterolemia, the present study investigated a possible influence of 17β-estradiol on aortic permeability to LDL. Twenty rabbits were initially ovariectomized and then fed a nonatherogenic diet for 10 weeks. One group of rabbits (n=10) received 4 mg of 17β-estradiol orally per day; the other group (n=10) received placebo. Serum concentrations of very-low-density lipoprotein cholesterol and triglycerides increased significantly more in the placebo group than in the estrogen group (P<.03), whereas there were no statistically significant differences between groups in LDL, high-density lipoprotein, or total cholesterol. At the end of the experiment, 125I-LDL was injected intravenously into each rabbit. Aortas were removed 3 hours later, and the aortic permeability to LDL was calculated from the radioactivity in the plasma and the aortic intimae/inner media. The aortic permeability to LDL was virtually identical in the 17β-estradiol (31.6±7.2 nL • cm⁻² • h⁻¹) and the placebo (36.9±7.9 nL • cm⁻² • h⁻¹) groups (mean±SEM). The aortic cholesterol content was also similar in the two groups. These data suggest that the plasma lipid-independent antiatherogenic effect of estradiol is not mediated through an effect on aortic permeability to LDL but rather is related to the metabolism of the lipoproteins after they have entered the arterial wall. (Arterioscler Thromb. 1994;14:243-247.)

Key Words • aortic permeability • atherosclerosis • estradiol • LDL • rabbits

Cardiovascular disease (CVD) is a major cause of death and morbidity in postmenopausal women in Western societies.1 Female sex hormones may have an important impact on CVD; this contention is supported by the increased risk of CVD in ovariectomized women, and presumably also in women after natural menopause, compared with age-matched menstruating women.2-4 Several human epidemiological studies indicate that estrogen replacement therapy reduces the risk of CVD.5-7 Recent studies with different animal models support an antiatherogenic effect of estradiol.8-10 They indicate that this beneficial effect is only partially explained by changes in serum lipids and lipoproteins, thus implying a direct effect of estrogen on the arterial wall.8-10 The underlying mechanisms remain to be elucidated.

Aortic permeability to low-density lipoprotein (LDL) in normocholesterolemic rabbits has recently been reported to be a strong predictor for the development of subsequent atherosclerosis during hypercholesterolemia.11 It may thus be speculated that estradiol replacement therapy could decrease the aortic permeability to atherospecific lipoproteins and thereby reduce the transport of cholesterol from plasma into the arterial intima, with inhibition of foam cell formation and atherosclerosis as a result. To investigate this hypothesis further, we studied the impact of 17β-estradiol on serum lipids, lipoproteins, and aortic permeability to LDL in normocholesterolemic, ovariectomized rabbits.

Methods

The animals were 10 pairs of sexually mature, female, white twin rabbits (each pair having different mothers) of the Danish Country strain, obtained from Statens Serum Institute, Copenhagen, Denmark. They were housed individually at the Panum Institute, University of Copenhagen, in stainless-steel cages at a room temperature of 20±2°C, a humidity of 50±10%, and a 12-hour light cycle. The study ran for 10 weeks.

In week 1, the rabbits were anesthetized with 0.4 mL/kg Hypnorm (0.2 mg/mL fentanyl plus 10 mg/mL fluanisone, Janssen Pharmaceutica NV, Beerse, Belgium) and underwent a bilateral ovariectomy.8 These procedures were in accordance with institutional guidelines. In week 2, each pair of twins was randomly assigned to receive oral daily treatment with either 4 mg of 17β-estradiol (one rabbit) or placebo (the twin). All rabbits were fed 80 g/d chow, which consisted of hormones (Schering AG, Germany) or placebo, corn oil 3.2 g (BP 80, Mecobenzon, Copenhagen), and standard rabbit pellets (Superfoss, Denmark), as described in detail elsewhere.9 The rabbits had free access to water.

Blood samples for serum triglycerides, total cholesterol, very-low-density lipoprotein (VLDL), LDL, high-density lipoprotein (HDL), and estradiol were taken from an ear vein before the ovariectomy, in weeks 5 and 9, and at the end of the study. To separate VLDL, LDL, and HDL, serum samples were adjusted to densities of 1.019 and 1.063 g/mL, respectively, and centrifuged at 4°C at 1.58×10⁷×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 ultracentrifuged fractions was determined enzymatically. Serum triglycer-
Aortic Cholesterol

The cholesterol content was determined in the intima/inner media of the aortic arch and thoracic aorta after extraction by the Lieberman-Burchard method.

Protocol for Labeling of LDL and Determination of Permeability

So that standardized LDL could be used, blood was taken from the same normocholesterolemic human donor in tubes containing Na2EDTA (2 mg/mL), chloramphenicol (40 μg/mL), gentamicin sulfate (0.1 mg/mL), e-amino-n-caproic acid (2.6 mg/mL), benzamidine (10 μg/mL), and aprotinin (10 kallikrein units/mL) (all from Sigma, Copenhagen). After sequential ultracentrifugation (1.58x10^8 g/cm^2 min) at densities of 1.019 and 1.063 g/mL, the isolated LDL fraction was recentrifuged to remove contaminating particles of density >1.063 g/mL (mostly albumin). The protein concentration of the LDL-containing fraction was measured at 220 nm.

LDL was iodinated by a modification of McFarlane's method. Labeled LDL solutions containing 8 μg of protein were adjusted to a pH of 10 with glycine buffer (1 mM/L) and mixed with 260 to 300 μCi of 125I (Amersham, Birkeroed, Denmark) before iodine monochloride was added. Unbound iodine was removed with prepacked PD-10 columns (Pharadex G-25M, Pharmacia, Copenhagen) before addition of rabbit albumin (160 mg/mL) and the LDL solution was dialyzed overnight at 4°C against saline (0.9%). LDL preparations used for injections were filtered through a 0.45-μm filter (Millipore GS 0.45, Millipore, France) before injection. 125I-LDL was injected into a lateral ear vein, followed by 2 mL of saline.

Blood samples were taken from the contralateral ear vein before injection of 125I-LDL and at 10, 30, 60, 120, and 180 minutes. Aortic permeability to LDL in the aortic arch was determined immediately after the last blood sample (180 minutes): the rabbits were anesthetized with intravenous injections of 5% pentobarbital sodium (50 mg/kg). The aorta was opened longitudinally and the surface rinsed with saline. The tissue was fixed with a perfusion fixative and then cut into small pieces, processed, and embedded in paraffin. The intima and media were stained with hematoxylin and eosin, and the number of LDL-filled gaps was counted.

Quality Assurance

The protein-bound radioactive fraction of the dose material, blood samples, and aortic tissues was determined by extraction with 2 mL trichloroacetic acid (TCA) at 4°C at a final concentration of 15% wt/vol after the addition of 100 ng of bovine albumin. After low-speed centrifugation, 125I was measured in 19 rabbits, as visualized in Fig 2, which also shows the changes in serum lipids, lipoproteins, and estradiol during the study. Triglycerides and VLDL cholesterol decreased in the estradiol-treated rabbits, as opposed to an increase in the placebo group (P<.03). There was a tendency toward a more pronounced increase in total cholesterol and LDL cholesterol in the placebo group than in the estrogen group (Fig 1). Fig 1 also shows that serum estradiol increased significantly more in the hormone group than in the placebo group (P<.001).

Aortic permeability to LDL in the aortic arch was measured in 19 rabbits, as visualized in Fig 2, which also shows the decrease in plasma radioactivity, the serum concentration of LDL cholesterol at the time of the permeability experiment, and the influx of LDL cholesterol into the aortic arch. There were no statistically significant differences between the two groups regarding initial body weight, serum estradiol, serum lipids, and lipoproteins as well as gain in body weight (Table). All rabbits in both groups ate all the chow. Fig 1 shows the changes in serum lipids, lipoproteins, and estradiol during the study. Triglycerides and VLDL cholesterol decreased in the estradiol-treated rabbits, as opposed to an increase in the placebo group (P<.03). There was a tendency toward a more pronounced increase in total cholesterol and LDL cholesterol in the placebo group than in the estrogen group (Fig 1).

Initial Characteristics of the Two Groups and Gain in Body Weight During the 10-Week Study

<table>
<thead>
<tr>
<th></th>
<th>Estradiol (n=9)</th>
<th>Placebo (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>2.9±0.1</td>
<td>2.9±0.1</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>2.3±0.3</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>0.7±0.2</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Serum VLDL cholesterol, mmol/L</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Serum LDL cholesterol, mmol/L</td>
<td>1.4±0.2</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>Serum HDL cholesterol, mmol/L</td>
<td>0.6±0.07</td>
<td>0.7±0.03</td>
</tr>
<tr>
<td>Serum estradiol, pmol/L</td>
<td>84±3.5</td>
<td>82±10</td>
</tr>
<tr>
<td>Body weight gain, kg</td>
<td>0.3±0.03</td>
<td>0.2±0.03</td>
</tr>
</tbody>
</table>

VLDL indicates very-low-density lipoprotein; LDL, low-density lipoprotein; and HDL, high-density lipoprotein. Values are mean±SEM.

and 94.3±0.9% of the plasma radioactivity was in the LDL fraction in the placebo and estradiol groups, respectively. About 98% was precipitable in TCA.

The aortic permeability to 125I-LDL was calculated as the aortic radioactivity divided by the area under the curve of plasma radioactivity versus time (the TCA-precipitable values were used).}

Statistics

Comparisons between the two groups were done by t tests, whereas relations between two parameters were expressed as parametric coefficients of correlation. The procedures of the SAS Institute were used for statistical analysis.

Results

One rabbit in the estradiol group had to be killed in week 2 because of a fractured lumbar vertebra, which left 19 rabbits in the study. There were no statistically significant differences between the two groups regarding initial body weight, serum estradiol, serum lipids, and lipoproteins as well as gain in body weight (Table). All rabbits in both groups ate all the chow. Fig 1 shows the changes in serum lipids, lipoproteins, and estradiol during the study. Triglycerides and VLDL cholesterol decreased in the estradiol-treated rabbits, as opposed to an increase in the placebo group (P<.03). There was a tendency toward a more pronounced increase in total cholesterol and LDL cholesterol in the placebo group than in the estrogen group (Fig 1). Fig 1 also shows that serum estradiol increased significantly more in the hormone group than in the placebo group (P<.001).

Aortic permeability to LDL in the aortic arch was measured in 19 rabbits, as visualized in Fig 2, which also shows the decrease in plasma radioactivity, the serum concentration of LDL cholesterol at the time of the permeability experiment, and the influx of LDL cholesterol into the aortic arch. There were no statistically significant differences between the estradiol and placebo groups in these parameters, although the combined effect of the nonsignificant reductions in aortic permeability (14%) and LDL cholesterol (29%) in the estradiol group resulted in a 44% reduction in the influx of LDL cholesterol from plasma into the aortic arch intima/inner media. Permeability within the pairs was not significantly related (Pearson correlation coefficient, r= .35; NS). The cholesterol content of the aortic arch
A VLDL-cholesterol (mmol/l)  
0.3-1  
0.0-0.6  
p = 0.02  

A LDL-cholesterol (mmol/l)  
1.2-0.6  
0.0-0.4  
p = 0.21  

A HDL-cholesterol (mmol/l)  
0.4-0.2  
0.0-0.0  
p = 0.84  

A Estradiol (pmol/l)  
60.0-40.0  
0.0-0.0  
p < 0.0003  

FIG 1. Graphs showing the changes in lipids, lipoproteins, and estradiol during the study period in the 17β-estradiol (•) and placebo (○) groups (mean±SEM). The mean changes in the two groups were compared by t tests. VLDL indicates very-low-density lipoprotein; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

Discussion

The two groups in the present study were well matched with respect to initial body weight and serum lipids. All rabbits were apparently healthy, gained in body weight during the study, and ate the supplied chow. The estradiol group achieved the expected serum concentrations of estradiol.2 These findings support the validity of the present study.

The decrease in serum triglycerides and VLDL cholesterol in the estradiol group and the tendency toward a smaller increase in total cholesterol and LDL cholesterol agree with previous findings in rabbits.3 These differences may be ascribed in part to an estrogenic induction of mRNA for the hepatic LDL receptor, thus resulting in increased LDL receptor levels and enhanced VLDL clearance.15,16 Those findings and the neutral effect on HDL cholesterol are, however, not exactly the same as those of women receiving postmenopausal hormone replacement therapy.17 In women receiving estrogen replacement therapy, HDL cholesterol usually increases and LDL cholesterol decreases.18 The present study examined the interaction between LDL and the arterial wall to achieve a better understanding of possible mechanisms behind the reported antithrombotic effect of estrogen.6,9 A recent study with a protocol similar to the present one found aortic permeability to LDL in normocholesterolemic rabbits to be a very strong predictor for subsequent development of atherosclerosis during hypercholesterolemia.11 The aortic arch permeability to LDL in that study was, on average, 32.1±6.6 nL · cm⁻² · h⁻¹ (mean±SEM, n=11), which is very similar to the values obtained in the present study. We used siblings, because a previous report19 demonstrated a close association between aortic permeabilities in male siblings (r = .67). This finding was not confirmed in the present study, and we were therefore unable to use the planned “paired” design (difference between sisters) to study the impact of estriadiol on aortic permeability. The present randomized, controlled study did not find a significant reduction in aortic permeability to LDL, although a tendency toward reduced permeability in the estrogen-treated group was found. The risk of overlooking a difference of 16.5 nL · cm⁻² · h⁻¹ (50%) in aortic permeability to LDL between the estrogen and placebo groups was estimated to be <15%. It is assumed that the iodinated human
LDL particle traces the permeability of the arterial wall also to the rabbit's endogenous LDL particles. It has previously been shown that the labeled protein moiety of rabbit LDL enters the arterial wall together with the labeled cholesteryl ester moiety in accordance with an entry of the intact LDL particle. In that study, the protein and lipid moieties were both labeled in vivo. We have recently compared the transfer of iodinated human LDL with cholesteryl ester-labeled human LDL into aortas of pigs and observed a high degree of similarity between the two values (unpublished data). We therefore consider it likely that the iodinated human LDL monitors a biologically important property of the arterial wall, its macromolecular permeability, and does not reflect the interaction between denatured apoprotein B and the arterial wall.

We therefore used the product of the aortic permeability to iodine-labeled LDL and the plasma LDL cholesterol concentration to estimate the influx of LDL cholesterol from plasma into the arterial intima: the LDL cholesterol influx was reduced by 29 pmol • cm⁻² • h⁻¹ in the estradiol-treated rabbits compared with controls. Assuming a constant LDL cholesterol concentration and LDL permeability throughout the 10-week experiment, this nonsignificant reduction would reduce the delivery of LDL cholesterol to the arterial intima/inner media by =50 nmol/cm² (29 pmol • h⁻¹ • cm⁻² • 24 hours • 7 days/wk • 10 weeks). However, the difference between the two groups in aortic arch cholesterol was only 9 nmol/cm² (NS). These calculations, although speculative, suggest that not only the influx of LDL cholesterol but probably also subendothelial metabolism and efflux of LDL cholesterol back to plasma may be important in the process of early atherosclerosis.

Previous human and animal studies have suggested that changes in plasma lipids can only explain about half of the antiatherogenic effect of estrogen. Accordingly, a recent important study in monkeys has suggested an effect of estradiol on aortic LDL metabolism. That study found an inhibitory effect of estrogen on the sum of aortic accumulation of iodinated tyramine-cellobiose-labeled LDL and its degradation products, which are trapped in the arterial wall. The lower value can be explained either by a lower influx of LDL from the plasma into the wall or by a higher efflux of LDL from the wall and back to the plasma. If the influx—as suggested by our study—is unaffected by estrogen, the findings by Wagner et al are compatible with an increased efflux of LDL from the aorta of estrogen-treated animals compared with control animals. This effect could be brought about if estrogen treatment alters the structure of the intercellular matrix, e.g., glucosaminoglycans, collagen, and elastin in the arterial wall, in a way that counteracts the binding of LDL to the matrix. A shorter transit time of LDL in the arterial wall may decrease the possibility of LDL oxidation or other chemical modifications that enhance foam cell formation. Alternatively, estrogen may directly inhibit LDL oxidation in the arterial wall and thereby decrease cholesterol uptake in macrophages, leading to reduced foam cell formation.
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Aortic permeability to LDL during estrogen therapy. A study in normocholesterolemic rabbits.

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